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Targeting B7x and B7-H3 as New Immunotherapies for Prostate Cancer

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14. ABSTRACT

We have made excellent progress during the three-year funding period, as evidenced by eight publications. We have shown the mechanisms by which B7x inhibits T cell function and promotes prostate cancer progress. We have solved structure of B7x IgV function domain and developed new mAbs to the IgV domain for a new cancer immunotherapy targeting B7x. Similarly, we developed new mAbs to the IgV domain of B7-H3 and are in the process of developing new cancer immunotherapy targeting B7-H3. We have discovered HHLA2 and TMIGD2 as the newest members of the B7-CD28 immune checkpoint family and further showed HHLA2 was highly expressed in human prostate cancer, which provided a potential new therapeutic target for human prostate cancer and other cancers as well.

Our research has won attention from biopharmaceutical industry. Our school licensed our B7x IPs to a start-up drug company and most likely will license our future B7-H3 IP to a well-established drug company, which provided the foundation for future clinical trials in patients with prostate cancer and other cancers.

15. SUBJECT TERMS

B7x, B7-H3, HHLA2, TMIGD2, Receptors, Immune Checkpoint, Prostate Cancer, Monoclonal Antibodies, Crystal Structure, Immunotherapy, T Cells,

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1. INTRODUCTION

Prostate cancer ranks second to lung cancer in terms of annual mortality among men in the United States. The mainstay of therapy for advanced prostate cancer, unchanged since 1941, continues to be palliative androgen deprivation. As such, more effective treatments of prostate cancer are urgently needed. In this respect, immunotherapy could offer an important alternative or adjunct to current therapy.

T cells of the immune system are the major combatants against cancers. One of the major goals of immunologic approaches for the treatment of cancer is the induction of cancer-specific T cell responses of sufficient magnitude to eliminate the cancer and prevent its recurrence. Despite some successful attempts by different workers in this area, tumor immunotherapy remains largely anecdotal and the collective experience has been frustrating. One reason for this less than optimal outcome is that, until recently, there was insufficient knowledge of normal regulatory processes that limit T cell function.

B7/CD28 family molecules control activation and function of T cells. We and others have discovered new members of the B7 family including B7x and B7-H3, and found that B7x inhibits T cell function. We completed a comprehensive investigation of B7 family molecules in a cohort of 823 patients who underwent prostatectomy for prostate cancer and have been followed for more than 7 years. This study revealed that patients with strong tumor B7x and B7-H3 expression were more likely to have disease spread at the time of surgery, and increased risk of cancer recurrence and cancer-specific death. These observations suggested that B7x and B7-H3 are exploited by prostate cancer as immune evasion pathways to inhibit T cell function. Therefore, we hypothesized that blockade of B7x and B7-H3 generates therapeutic tumor immunity against prostate cancer. The first aim of this proposal is to develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3. The second aim of this proposal is to examine combination therapy of anti-B7x/anti-B7-H3 with other therapies. The proposed research is significant, because it is anticipated to provide new targets for therapeutic interventions that will aid the growing numbers of prostate cancer patients.

2. KEYWORDS

B7x, B7-H3, HHLA2, TMIGD2, Receptors, Immune Checkpoint, Prostate Cancer, Monoclonal Antibodies, Crystal Structure, Immunotherapy, T Cells,

3. ACCOMPLISHMENTS

What were the major goals of the project?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3. (Months 1-36)

1a. Generation of mAbs to human and mouse B7-H3 (Months 1-12).
1b. Effectiveness of B7x and/or B7-H3 blockade in a subcutaneous prostate tumor model (Months 6-24).
1c. Effectiveness of B7x and/or B7-H3 blockade in treatment of prostate tumor metastasis (Months 12-30).
1d. Effectiveness of B7x and/or B7-H3 blockade in treatment of primary prostate tumor (Months 12-36).
1e. Effect of B7x- or B7-H3-specific mAbs on T cell function in vivo in humanized NSG mice. (Months 12-24).

Task 2. Examine combination therapy of anti-B7x/anti-B7-H3 with other therapies. (Months 12-36).

2a. Synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4 (Months 18-30).
2b. Synergy between B7x/B7-H3 blockade and regulatory T cell depleting (Months 18-30).
2c. Potential mechanism of anti-B7x/B7-H3 therapy: blockade of B7-mediated T cell immunosuppression (Months 12-36).
2d. Potential mechanism of anti-B7x/B7-H3 therapy: effect on the generation of induced regulatory T cells (iTreg) and myeloid-derived suppressor cells (MDSCs) (Months 12-36).
2e. Potential mechanism of anti-B7x/B7-H3 therapy: antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (Months 12-30).

What was accomplished under these goals?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3. (Months 1-36)

We first solved crystal structure of the human B7x IgV domain. Like other B7 family members, B7x possesses extracellular B7x immunoglobulin variable (IgV) and immunoglobulin constant (IgC) domains. The IgV domain has previously been characterized as the receptor-binding domain for B7-1, B7-2, PD-L1, and PD-L2. Therefore, we sought to understand the structure of the B7x IgV domain (B7x-IgV) to inform future studies of its interaction with receptors and antibodies. We successfully determined the crystal structure of the human B7x-IgV domain at 1.59 A° resolution. We generated B7x gene knock-out mice. Therefore we took advantage of these mice and

the B7x crystal structure information for generating mAbs against both human and mouse B7x I. B7x knock-out mice were immunized with human B7x-IgV protein. After 3 weeks, the mice were immunized with mouse B7x-IgV protein with hopes to produce cross-reactive antibodies. We generated many hybridoma clones and finally selected seven for further studies. By Surface Plasmon Resonance we determined these seven clones had very high affinity with KD between 0.01-8.8 nM. We further determined the sequences of VH and VL of all seven mAbs. We developed an in vitro function screen system with APC/T cells and found mAb H19 and H17 had the best abilities to neutralize the human B7x-mediated human T cell co-inhabitation. We then treated mice bearing prostate cancer or other type of cancer with these mAbs and found some of them inhibited tumor growth. Our school licensed all our B7x IPs (cancer immunotherapy and autoimmune disease immunotherapy) to a start-up drug company. Our mAbs will be humanized for future potential IND and clinical trials in cancer patients including prostate cancer.

We previously solved crystal structure of the human B7-H3 IgV-IgC domain. During the three-year funding period, we took advantage of the B7-H3 crystal structure information and B7-H3 gene knock-out mice for generating mAbs against both human and mouse B7-H3. We generated many hybridoma clones and finally selected ten for further studies. By Surface Plasmon Resonance we determined these ten clones had high affinity. We then treated mice bearing prostate cancer or other type of cancer with these mAbs and found some of them inhibited tumor growth. We will further determine the sequences of VH and VL of some mAbs in order to humanize one or two best mAbs.

To facilitate future clinical trials, we have spent two years (year 2 and 3) to examine the effect of B7x- or B7-H3-specific mAbs on T cell function in vivo in humanized NOD-scid IL2Rg-/- mice (NSG). We have successfully established humanized NSG mice for prostate cancer immunotherapy. NSG mice were first engrafted with human prostate cancer lines and then human peripheral blood mononuclear cells. These humanized mice usually develop T cell-mediated graft-versus host immune responses within one month, so human T cells are activated and express receptors for B7x and B7-H3 and human prostate cancer cells express B7x or B7-H3. Once we obtain humanized mAbs against B7x and B7-H3, we will use our humanized NSG-bearing human prostate cancers to examine if our anti-B7x and/or anti-B7-H3 enhance T cell function and reduce prostate cancer growth in vivo.

Task 2. Examine combination therapy of anti-B7x/anti-B7-H3 with other therapies (Months 12-36).

We screened a panel of mAbs against PD-1 or CTLA-4 for in vivo immunotherapy. Our results showed that clone RMPI–14 of anti-PD-1 and clone 9H10 of anti-CTLA-4 worked well in vivo. Therefore, we are performing experiments to see if there are synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4. We performed experiments to dissect the potential mechanisms of anti-B7x/B7-H3 therapy. We found that B7x affected the differentiation of naïve CD4 T cells into Th1 (CD3/CD28 + anti IL-4 + IL-2) and Th17 (CD3/CD28 + IFNg + IL-4 + IL-6 + TGF beta), but not Th2 (CD3/CD28 + anti IFNg + IL-4). In in vivo tumor model, we found expression of B7x on tumor cells significant increased tumor burden in the lung, which was marked by a significant increase in M2 tumor associated macrophages and antigen-specific

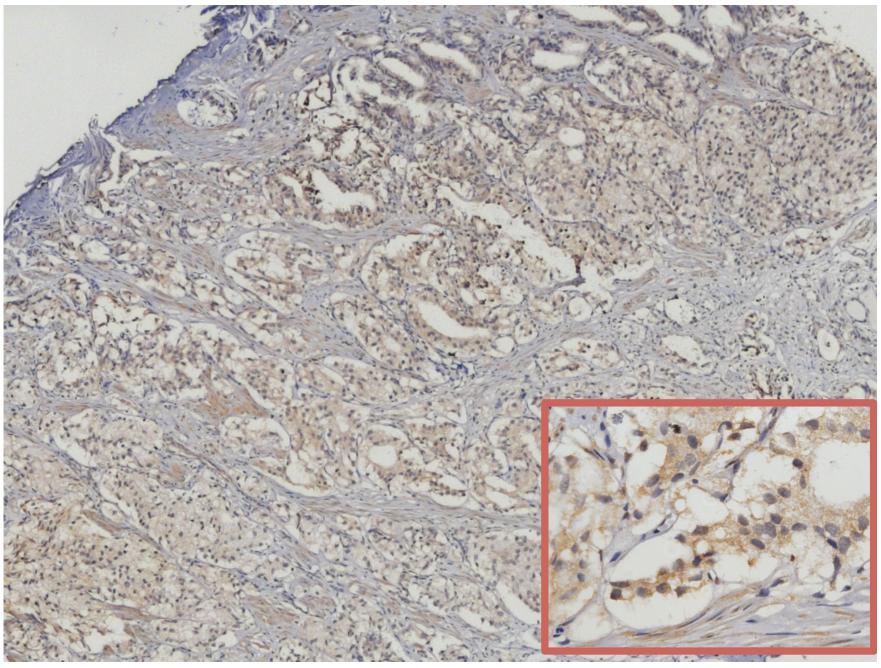
CD8 T cell exhaustion.

We examined if pro-inflammatory and anti-inflammatory cytokines could change B7x expression. We showed that pro-inflammatory and anti-inflammatory cytokines IFNγ, TNFα, and IL-10 did not induce expression of B7x on human or murine cancer cells. These results suggest that the tumor microenvironment, rather than a single cytokine, is the key to up-regulate B7x expression on tumor cells. We will dissect which factor(s) within the tumor microenvironment are responsible for the enhanced B7x expression on cancer cells in vivo.

One of the key challenges in the field is to find receptors for B7x and B7-H3. Recently a patent application suggested that B7x interacted with Neuropilin-1 (NRP1), therefore, we were intrigued and decided to evaluate the potential binding. However, after extensive experiments of both Bio-Layer Interferometry(BLI) assays and FACS assays, we did not find evidence for the direct interaction between B7x and NRP1, whereas NRP1 interacted with Sema3a and vEGF very well. Further studies are necessary to discover still unknown cognate receptor(s) for B7x and B7-H3.

Related work which were not in the initial proposal

We recently discovered HHLA2 as the newest B7 immune checkpoint and it was over-expressed in human prostate cancer. We generated new mAbs to HHLA2 and developed a new IHC protocol for HHLA2 protein expression. Interestingly enough, none of three normal prostate tissues expressed HHLA2, whereas three out of nine prostate cancer samples were HHLA2 positive (see Fig), suggesting the HHLA2 pathway represents a novel immunosuppressive mechanism within the tumor microenvironment of human prostate cancer and an attractive target for human prostate cancer immunotherapy.

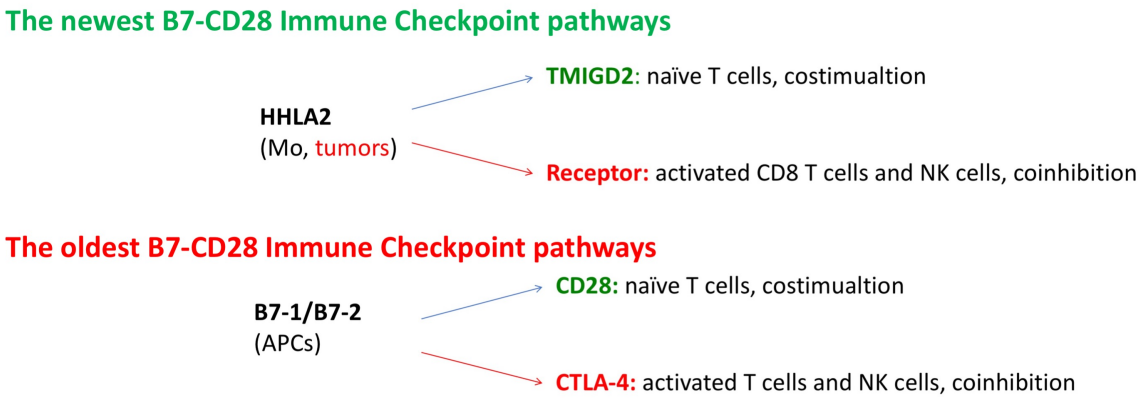


Unlike B7x and B7-H3 whose receptors have not been found yet, we have quickly discovered two receptors for HHLA2: TMIGD2 and R2. We found that HHLA2

was expressed in human and monkey but not in mouse and rat, and hypothesized that its receptors should have the same expression patter as ligand and receptor often show co-evolution. Through bioinformatics analysis/immunological approach/high-throughput screening, we first identified Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) as a receptor for HHLA2 in 2015. This year (2017) we further identified R2 as the second receptor for HHLA2.

In sum, we have discovered the newest B7-CD28 immune checkpoint pathways: HHLA2/TMIGD2/R2. The comparison between this newest pathways with the oldest B7-CD28 immune checkpoint pathways B7-1/B7-2/CD28/CTLA-4 suggests these two pathways have some similarity but yet difference (see Fig). Due to their fundamental biological importance and therapeutic potential, there has been considerable interest in the identification of additional molecules with costimulatory or coinhibitory function. we now have discovered the newest the newest B7-CD28 immune checkpoint pathways which is not expressed in three normal prostate tissues but expressedin three out of nine prostate cancer samples. This unique human T-cell coinhibitory pathway may afford unique strategies for the treatment of human cancers, autoimmune disorders, infection, and transplant rejection and may help to design better vaccines.

The working model: HHLA2 and receptors



What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?

Our research has won attention from biopharmaceutical industry. Our school licensed our B7x IPs to a start-up drug company and most likely will license our future B7-H3 IP to a well-established drug company, which provided the foundation for future clinical trials in patients with prostate cancer and other cancers.

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS

Nothing to Report

6. PRODUCTS

Journal publications:

Jeon H, Vigdorovich V, Garrett-Thomson SC, Janakiram M, Ramagopal UA, Abadi YM, Lee JS, Scandiuzzi L, Ohaegbulam KC, Chinai JM, Zhao R, Yao Y, Mao Y, Sparano JA, Almo SC, **Zang X**. Structure and cancer immunotherapy of the B7 family member B7x. Cell Reports, 6:1089-1098, 2014

Ohaegbulam KC, Assal A, Lázár-Molnár E, Yao Y, **Zang X**. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. Trends in Molecular Medicine, 21:24-33, 2015

Janakiram M, Chinai JM, Fineberg S, Fiser A, Montagna C, Medaverepu R, Castano E, Jeon H, Ohaegbulam KC, Zhao R, Zhao A, Almo SC, Sparano JA, **Zang X**. Expression, clinical significance, and receptor identification of the newest B7 family member HHLA2 protein. Clinical Cancer Research, 21:2359-2366, 2015

Janakiram M, Chinai JM, Zhao A, Sparano JA, **Zang X**. HHLA2 and TMIGD2: New immunotherapeutic targets of the B7 and CD28 families. OncoImmunology, 4: e1026534-1- e1026534-3, 2015

Chinai JM, Janakiram M, Chen F, Chen W, Kaplan M, **Zang X**. New Immunotherapies targeting the PD-1 pathway. Trends in Pharmacological Sciences, 36:587-595, 2015

Liu W, Almo SC, **Zang X**. Co-stimulate or co-inhibit regulatory T cells, which side to go? Immunological Investigations, 45:813-831, 2016

Picarda E, Ohaegbulam KC, **Zang X**. Molecular pathways: Targeting B7-H3 (CD276) for human cancer immunotherapy. Clinical Cancer Research, 22:3425-3431, 2016

Janakiram M, Shah UA, Liu W, Zhao A, Schoenberg MP, **Zang X.** The third group of the B7-CD28 immune checkpoint family: HHLA2, TMIGD2, B7x and B7-H3. Immunological Reviews, 276:26-39 , 2017

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

No change

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

Jeon H, Vigdorovich V, Garrett-Thomson SC, Janakiram M, Ramagopal UA, Abadi YM, Lee JS, Scandiuzzi L, Ohaegbulam KC, Chinai JM, Zhao R, Yao Y, Mao Y, Sparano JA, Almo SC, **Zang X**. Structure and cancer immunotherapy of the B7 family member B7x. Cell Reports, 6:1089-1098, 2014

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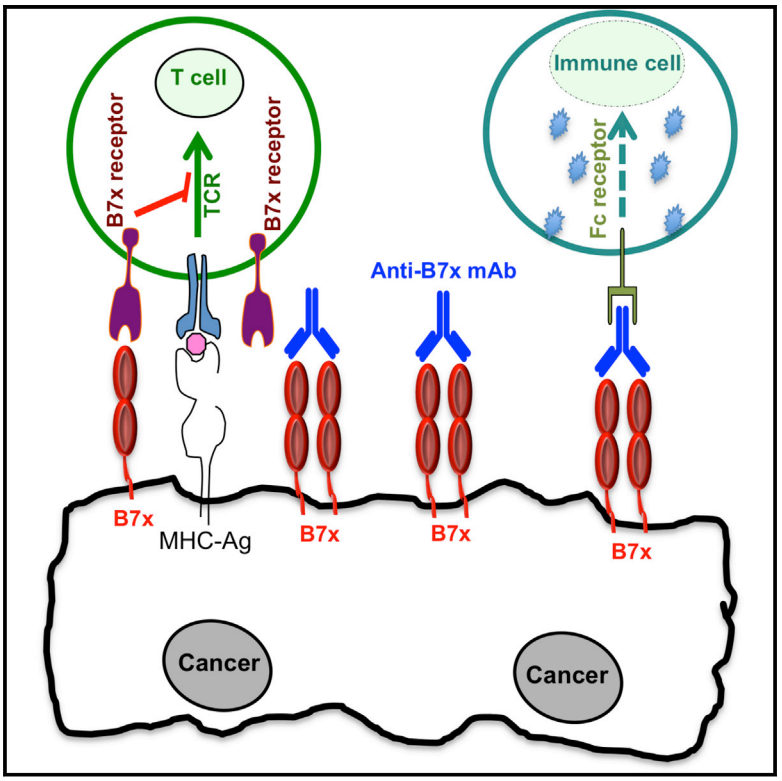
Article

Cell Reports

Structure and Cancer Immunotherapy of the B7 Family Member B7x

Graphical Abstract



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In Brief

B7x is an attractive therapeutic target for cancer immunotherapy. Jeon et al. now develop an in vivo screening system to find therapeutic monoclonal antibodies (mAbs) that target B7x on tumors. The mAb 1H3 is able to significantly inhibit growth of B7x-expressing tumors in vivo through multiple mechanisms.

Highlights

The crystal structure of the human B7x IgV domain is determined

An in vivo system is developed to screen therapeutic mAbs against B7x

mAb 1H3 treatment suppresses tumor growth and prolongs survival in tumor models

Anti-B7x immunotherapy creates an environment with enhanced antitumor immunity

Accession Numbers

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Cell Reports
Article

Structure and Cancer Immunotherapy of the B7 Family Member B7x

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SUMMARY

B7x (B7-H4 or B7S1) is a member of the B7 family that can inhibit T cell function. B7x protein is absent in most normal human tissues and immune cells, but it is overexpressed in human cancers and often correlates with negative clinical outcome. The expression pattern and function of B7x suggest that it may be a potent immunosuppressive pathway in human cancers. Here, we determined the crystal structure of the human B7x immunoglobulin variable (IgV) domain at 1.59 Å resolution and mapped the epitopes recognized by monoclonal antibodies. We developed an in vivo system to screen therapeutic monoclonal antibodies against B7x and found that the clone 1H3 significantly inhibited growth of B7x-expressing tumors in vivo via multiple mechanisms. Furthermore, the surviving mice given 1H3 treatment were resistant to tumor rechallenge. Our data suggest that targeting B7x on tumors is a promising cancer immunotherapy and humanized 1H3 may be efficacious for immunotherapy of human cancers.

INTRODUCTION

T cell costimulation and coinhibition mediated by the B7 ligand family and the CD28 receptor family have crucial roles in modulating T cell activation, proliferation, and differentiation into effector function and memory generation (Greenwald et al., 2005; Zang and Allison, 2007). The B7-1/B7-2/CD28/CTLA-4 pathway is a well-characterized T cell costimulatory and coinhibitory pathway. A monoclonal antibody (mAb) against CTLA-4 was recently approved for the treatment of metastatic melanoma (Hodi et al., 2010; Sharma et al., 2011), and CTLA-4-Ig fusion protein has been used to treat rheumatoid arthritis and prevent acute kidney transplant rejection (Fiocco et al., 2008; Vincenti et al., 2011). The past decade has witnessed a new era in the discovery of other B7 and CD28 members and understanding of their immune regulation, including B7h/ICOS, PD-L1/PD-L2/PD-1, B7-H3/receptor, B7x/receptor, and HHLA2 (B7y/B7-H5/B7h7)/TMIGD2 (CD28h) (data not shown; Zhao et al., 2013; Zhu et al., 2013). mAbs against PD-1 and PD-L1 are currently in clinical trials with cancer patients (Brahmer et al., 2012; Topalian et al., 2012). Clearly, further studies of the less characterized B7/CD28 pathways will not only sharpen our understanding of the immune system but also lead to new therapies for a wide range of diseases.

B7x (B7-H4 or B7S1), a member of the B7 family, can inhibit T cell proliferation and cytokine production in vitro (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003). Recent studies reveal that overexpression of B7x on pancreatic cells is sufficient to abolish CD4 or CD8 T cell-induced diabetes (Lee et al., 2012; Wei et al., 2011), demonstrating that manipulating the B7x pathway can achieve significant functional consequences in vivo. In contrast to the expression pattern of B7-1 and B7-2, B7x protein is mainly detected in nonlymphoid organs (Hofmeyer et al., 2012; Lee et al., 2012; Tringler et al., 2005; Wei et al., 2011). One of the most intriguing characteristics of B7x is that it is overexpressed in numerous human cancers and, in many cases, correlates with negative clinical outcome (Barach et al., 2011; Janakiram et al., 2012; Zang and Allison, 2007). A large investigation of B7 family molecules in human malignancy demonstrated that prostate cancer patients with tumors that express B7x highly are more likely to have disease spread at the time of surgery and are at an increased risk of cancer recurrence and cancer-specific death (Zang et al., 2007). In another study, 103 ovarian cancer samples tested expressed B7x (Zang et al., 2010). In contrast to tumor tissues, only scattered B7x-positive cells are detected in nonneoplastic ovarian tissues (Zang et al., 2010). In line with these results, others have reported that B7x overexpression can be seen in human cancers of the lung (Sun et al., 2006), breast (Tringler et al., 2005), kidney (Krambeck et al., 2006), pancreas (Awadallah et al., 2008), esophagus (Chen et al., 2011), skin (Quandt et al., 2011), and gut (Jiang et al.,

Table 1. Data Collection, Phasing, and Refinement Statistics for SAD

Data Collection	Native	I3C
Space group	P 4_32_12	P 4_32_12
Cell dimensions		
α, β, γ (Å)	46.5, 46.5, 115.77	46.47, 46.47, 116.15
a, b, g (°)	90, 90, 90	90, 90, 90
Wavelength	1.075	1.5402
Resolution (Å)	43.1–1.59	38.72–1.79
R_{sym} or R_{merge}	8.8 (48.9)	16.1 (179)
I/σI	17.7 (1.6)	13.8 (1.8)
Completeness (%)	90.1 (100.0)	86.4 (89.4)
Redundancy	13.3 (13.5)	18.4 (19.7)
Refinement		
Resolution (Å)	1.59	
No. reflections	15,194	
R_{work}/R_{free}	17.4 (23.2)/19.2 (22.6)	
No. of atoms		
Protein	871	
Ligand/ion	61	
Water	111	
B-factors		
Protein	15.0	
Ligand/ion	13.8	
Water	24.1	
Rmsd		
Bond lengths (Å)	0.009	
Bond angles (°)	1.474	

One crystal was used for native and one for I3C-derivatized data sets. Values in parentheses are for the highest-resolution shell.

2010). In renal cell carcinoma (Krambeck et al., 2006), patients with tumors expressing B7x are three times more likely to die of cancer compared to patients lacking B7x. In esophageal squamous cell carcinoma, expression levels of B7x on tumor cells are significantly correlated with distant metastasis, tumor stage and worse survival and are inversely correlated with densities of CD3 T cells in tumor nest and CD8 T cells in tumor stroma (Chen et al., 2011).

The overexpression of B7x by so many types of human cancers suggests that this pathway may be exploited as an important immune-evasion mechanism. Here, we report the first crystal structure of the human B7x immunoglobulin variable (IgV) domain and developed a cancer immunotherapy using mAbs recognizing this domain. Our findings suggest that targeting B7x on tumors can be an innovative tumor immunotherapy.

RESULTS

Crystal Structure of the Human B7x IgV Domain

Like other B7 family members, B7x possesses extracellular IgV and immunoglobulin constant (IgC) domains (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003). The IgV domain has previously been characterized as the receptor-binding domain for B7-1 (Stamper et al., 2001), B7-2 (Schwartz et al., 2001), PD-L1 (Lin et al., 2008), and PD-L2 (Lázár-Molnár et al., 2008). Therefore, we sought to understand the structure of the B7x IgV domain (B7x-IgV) to inform future studies of its interaction with receptors and antibodies. Human and murine B7x sequences share ~90% sequence identity overall and in their IgV domains. The crystals of human B7x-IgV exhibited diffraction consistent with the space group $P4_32_12$ (a = 46.5 Å, b = 46.5 Å, c = 115.77 Å; one molecule per asymmetric unit) and extended to 1.59 Å (Table 1). The final model consists of residues 35–148, which are organized into a β sandwich composed of sheets ABED (back-sheet; light blue) and C′′C′CFG (front-sheet; light green) (Figure 1A). The sequence of the human B7x-IgV is predicted to contain a single glycosylation site at Asn112, and we observed a region of well-defined electron density near this position consistent with the presence of corresponding to five sugar residues of a branched glycan (Figure 1B). Superposition of B7x and PD-L1 IgV domains (Figure 1C) resulted in a root-mean-square deviation (rmsd) of 1.327 Å with differences apparent largely in the loop regions, demonstrating that the fold is highly conserved between these two B7 family members.

Interaction of mAbs with the B7x IgV Domain

We recently generated B7x-specific mAbs from $B7x^{-/-}$ mice (Wei et al., 2011). mAbs 1H3 (immunoglobulin G1 [IgG1]) and 12D11 (IgG1) bound human or mouse B7x, but not other B7 family members (Figure S1A). We estimated the binding rate constants and derived the corresponding equilibrium dissociation constants (K_D's) for the interactions of mAbs with recombinant murine B7x ectodomain, as well as murine and human B7x IgV through surface plasmon resonance (SPR). In these experiments, purified antibodies were immobilized on the biosensor surface and recombinant protein samples at various concentrations were injected for 120–240 s followed by dissociation in running buffer. mAbs 1H3, 12D11, and 15D12 strongly interacted with all of these proteins, and dissociation constants are summarized in Table 2. Representative sensorgrams are shown in Figure S1B.

Mapping the Epitopes in the Human B7x IgV Domain Recognized by 1H3 and 12D11

To define the epitope recognized by 1H3 and 12D11, we generated a series of B7x point mutants and measured their effect on binding of 1H3 and 12D11. Both antibodies showed a similar pattern of binding to B7x except in the case of the I62A mutant. Mutations at residues E59, I62, K63, E74, K84, F104, D106, Q107, and S135 resulted in greater than 40% loss of antibody binding (Figure 1D; Figure S1C), even though their overall expression was similar to wild-type B7x. These observations suggest that perturbation of these residues did not cause global B7x misfolding or instability and that the effects on antibody binding were the result of impaired/unfavorable interactions at the binding interface. Clustering of the identified residues on the surface of the B7x IgV domain further suggests that the observed effects on antibody binding were not due to nonspecific effects on overall structure or stability of B7x.

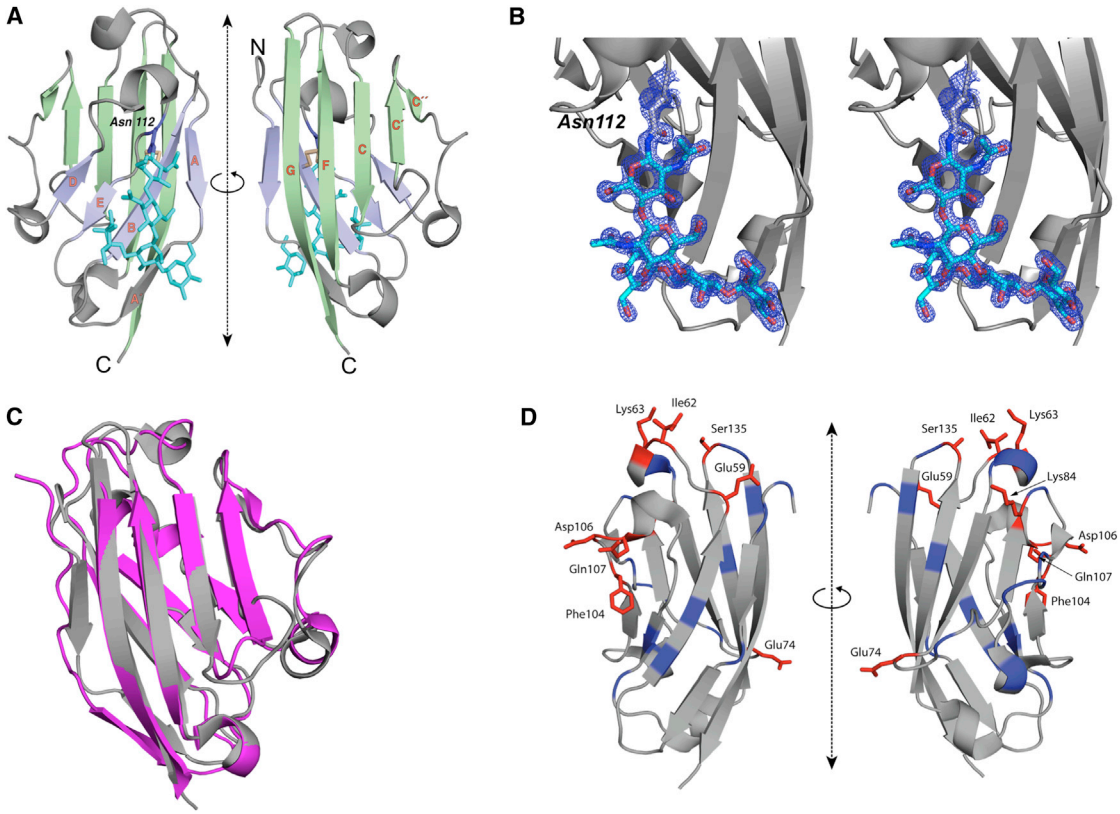

Figure 1. The Structure of the Human B7x IgV Domain and the Epitopes Recognized by Monoclonal Antibody against B7x

(A) Ribbon representation of the structure of human B7x-IgV (front sheet, light green; back sheet, light purple; disulfide, light yellow) is shown in two orientations. The strands of the β sandwich are labeled in orange; the side chain of Asn112 (purple) and the N-linked glycan (cyan) are shown in stick-figure representation.

(B) Electron density observed near Asn112 corresponds to an N-linked glycan. Ribbon representation of the structure of human B7x-IgV (gray) is shown. Electron density map ($2F_o - F_c$), contoured at +2σ, and covering the area near and including Asn112 is shown in mesh representation (blue). The five residues of N-linked glycan are shown in stick representation.

(C) Superposition of B7x and PD-L1. Gray represents the IgV domain of human PD-L1, and Pink represents the IgV domain of human B7x.

(D) Ribbon diagram of the IgV domain of B7x showing the location of residues targeted for mutagenesis. Positions that when mutated resulted in at least 40% reduction in 1H3 binding are highlighted in red, while the remainder of targeted positions are highlighted in blue.

Anti-B7x mAb Therapy in Mouse B7x-Expressing Tumor Models

Although B7x is overexpressed in many human cancers (Jeon et al., 2013), it is lost rapidly in vitro culture (Dangaj et al., 2013). We found that most human and mouse tumor cell lines were B7x protein negative. To develop a functional screening system for immunotherapy, we first established tumor cell lines that stably express cell-surface B7x using a retroviral expression vector transfection. (Figures S2A and S2B). The B7x expression level on CT26 cells was comparable to the expression levels of B7x on human cancer cell lines such as MCF-7, MDA-MB-468, and SK-BR-3 (Figures S2C–S2E). Mouse colon carcinoma CT26 and murine and human B7x/CT26 were intravenously (i.v.) injected into syngeneic BALB/c mice to induce experimental lung metastasis. The average number of lung tumor nodules in murine and human B7x/CT26 group was ~3.5-fold higher than that in the CT26 group (Figure S2F). In line with this result, B7x/CT26-injected mice had a lower survival rate compared to mice receiving naive CT26 cells (Figure S2G). These results demonstrate that B7x overexpression on a murine tumor cell line, like on human tumors, correlates with worse outcomes.

We next screened the in vivo therapeutic effect of B7x-specific mAbs in the B7x/CT26-induced pulmonary metastasis model. B7x/CT26 cells were i.v. injected into BALB/c mice followed by intraperitoneal (i.p.) injection of anti-B7x mAbs. On day 17, lung tumor nodules were examined. We found that two mAbs, 1H3 and 12D11, significantly reduced ~60% of tumor nodules in lungs (Figure 2A). The 4T1 murine tumor model is a widely used model in which the cancerous cells spontaneously metastasize to the lung (Aslakson and Miller, 1992). We then decided to validate the efficacy of 1H3 in the murine B7x/4T1 primary tumor model (Figure S2H). 1H3 treatment significantly suppressed primary tumor growth and efficiently reduced primary tumor-induced metastatic tumor nodules in the lung (Figures 2B and S4B). These results from two tumor models suggest that anti-B7x markedly reduces tumor nodules in lungs.

Table 2. Surface Plasmon Resonance Measurements

mAb	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
Murine B7x (IgV Domain)			
1H3	2.455(4) × 10^6	0.001248(7)	0.508(3)
12D11	2.140(4) × 10^6	0.001341(8)	0.627(4)
15D12	1.790(3) × 10^6	0.001135(7)	0.634(4)
Murine B7x			
1H3	6.71(3) × 10^5	0.001298(7)	1.94(1)
12D11	6.62(4) × 10^5	0.001177(7)	1.78(1)
15D12	4.40(3) × 10^5	0.001095(6)	2.49(2)
Human B7x (IgV Domain)			
1H3	2.53(2) × 10^5	0.00917(4)	36.2(3)
12D11	2.13(1) × 10^5	0.00928(3)	43.5(3)
15D12	1.388(7) × 10^5	0.01039(2)	74.9(4)

The value in parentheses denotes the SE in the last digit.

Anti-B7x mAb Therapy in a Human B7x-Expressing Tumor Model

B7x is very evolutionally conserved (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003), and mature B7x protein shares 90% amino acid identity in the extracellular domains between human and mice. Since both 1H3 and 12D11 recognized human B7x (Table 2), we wanted to test the therapeutic effects of these two mAbs in a human B7x-expressing tumor model in vivo. Like mouse B7x, the expression of human B7x on CT26 markedly increased tumor nodules in the lung (Figure S2F). We then examined the effect of mAbs on hB7x/CT26 in vivo. Mice were i.v. injected with hB7x/CT26 and then treated with 1H3 or 12D11. On day 17, the average numbers of lung tumor nodules in 1H3-treated and 12D11-treated groups were 97 and 236, respectively, whereas the number in the control group was 251 (Figure 2C). Because proteins in the tumor microenvironments can be modified by posttranslational modifications such as glycosylation and phosphorylation, we questioned whether 1H3 recognized human B7x that is naturally expressed in human tumors. Using immunohistochemical staining, we found that 1H3 recognized nature B7x in various human cancers from the colon, ovary, skin, lung, breast, liver, nasal cavity, liposarcoma, pancreas, and stomach (Figure 2D). These results suggest that human B7x promotes tumor growth in vivo and that mAb 1H3 recognizes human B7x and inhibits progression of these tumors in vivo. As 1H3 was the only mAb that inhibited both human and mouse B7x-mediated tumor progression, we used it for the subsequent experiments.

1H3 mAb-Treated Mice Survive Tumor Rechallenge

We next investigated the effect of 1H3 on the survival of mice bearing B7x/CT26 tumor. In agreement with the lung tumor nodule results, 1H3-treated mice had a significant lower mortality than did control immunoglobulin G (IgG)-treated mice. By day 60 postinjection of tumor, 100% of IgG-treated mice were dead, whereas only 50% of 1H3-treated mice had died (Figure 3A). These surviving mice appeared to be healthy. We then examined whether the surviving mice had resistance to the tumor rechallenge. These mice were rechallenged with the same number of B7x/CT26 cells, and all of them remained alive. On day 120, mice were sacrificed and their lungs were free of visible tumor nodules. Furthermore, hematoxylin and eosin (H&E) staining of lung sections from these mice showed that they were free of cancerous cells (Figure 3B).

Anti-B7x Therapy Increases Infiltrating T and NK Cells and Decreases Infiltrating MDSCs in Tumors

To dissect the therapeutic mechanisms of 1H3 treatment, we prepared single-cell suspensions from tumor-bearing lungs and analyzed the immune cells by flow cytometry. 1H3-treated mice had a significantly higher percentage of $CD45^+$ immune cell infiltrate than control IgG-treated mice (Figure 4A). Among these $CD45^+$ cells, the 1H3 treatment strongly increased tumor infiltrates of CD8 T cells and natural killer (NK) cells (Figure 4B), two major types of antitumor immune cells. We used SPSYVYHQF/H-$2L^d$ tetramer to detect CD8 T cells specific for CT26 tumor antigen epitope AH1 (amino acids 423–431 SPSYVYHQF) (Huang et al., 1996). In agreement with the increased total CD8 T cells, 1H3 treatment increased the percentage of AH1-specific CD8 T cells (Figure 4C). In addition, we found that the percentage of AH1-specific CD8 T cells in the blood was reduced in 1H3-treated mice (Figure 4D), but the percentage of tumor-associated macrophages in the blood was not changed by 1H3 treatment (Figure S3A). There were no significant changes in AH1-specific CD8 T cells and tumor-associated macrophages in spleens. These results suggest that 1H3 treatment facilitated the migration of tumor-specific CD8 T cells from the blood to tumor-bearing lungs. Recent studies identified the coexpression of Tim-3 and PD-1 (Tim-3+PD-1+) cells as a unique phenotype of exhausted CD8 or CD4 T cells in melanoma and leukemia (Fourcade et al., 2010; Goding et al., 2013; Zhou et al., 2011). Therefore, we examined the effect of 1H3 on these two inhibitory receptors on T cells. We found that 1H3-treated mice had significantly fewer CD4 T cells that were Tim-3+PD-1+, Tim-3+ alone and PD-1+ alone relative to those of control mice (Figure 4E). These results suggest that 1H3 treatment reduced the conversion of CD4 T cells from an activated to an exhausted state. Along with these findings, 1H3 treatment enhanced CD4 T cells to produce interferon-γ (IFN-γ; Figure 4F), a critical cytokine for antitumor immunity. Perforin and granzyme B are effector molecules in CD8 T cells and NK cells that mediate tumor cell death. We found that 1H3 treatment increased their production in CD8 T cells and NK cells, although the difference did not reach statistical significance (Figures S3B and S3C). In the tumor microenvironment, suppression of effector T cell function is often driven by immunosuppressive cells. Therefore, we investigated the effect of 1H3 treatment on immunosuppressive cell infiltrates in tumor-bearing lungs. We found that the treatment did not change the percentage of $Foxp3^+CD4^+$ regulatory T cells (Tregs; Figure S3D), but it did reduce $CD11b^+Ly6C^+$ monocytic myeloid-derived suppressor cells (MDSCs) infiltrating the tumor (Figure 4G). The increase of CD8 T cells, NK cells, and IFN-γ-producing CD4 T cells combined with the reduction of MDSCs in the 1H3 treatment collectively creates an environment with a lower ratio of suppressive cells to antitumor effector cells (Figure 4H).

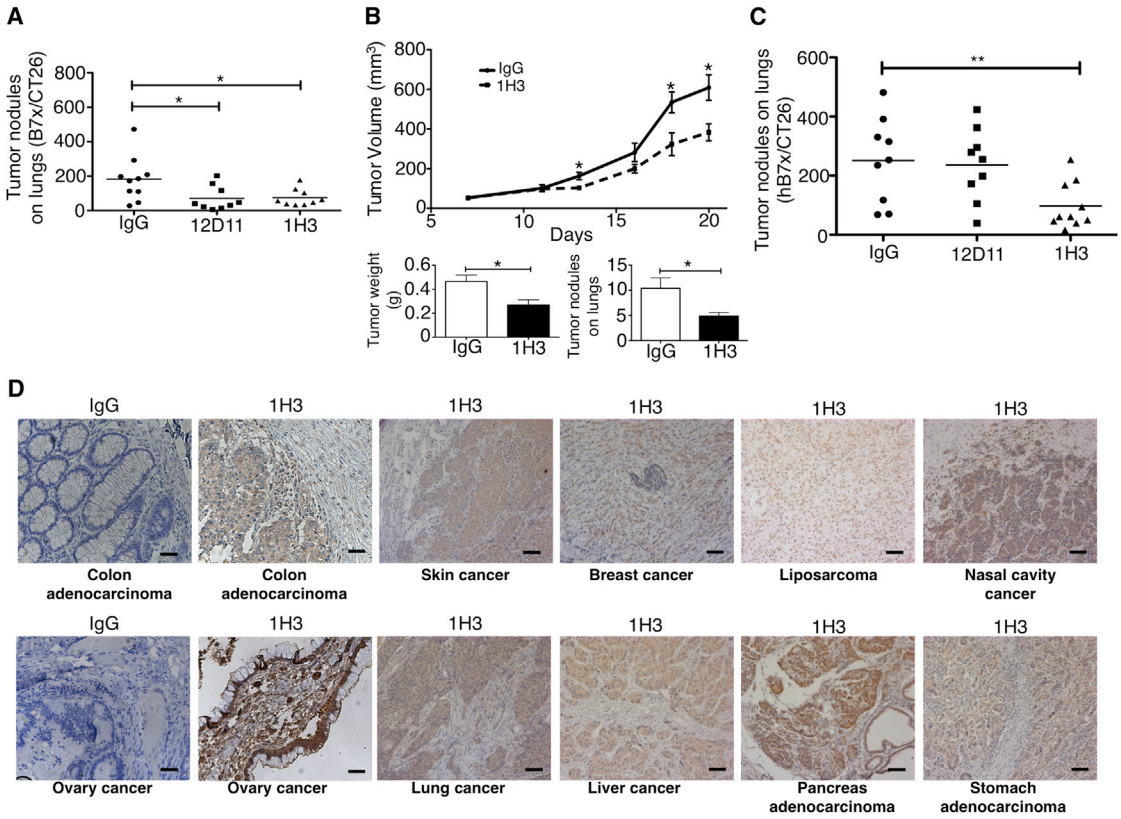

Figure 2. Effect of Anti-B7x Monoclonal Antibodies on Tumor Growth

(A) BALB/c mice were i.v. injected with B7x/CT26, then with anti-B7x mAbs 12D11 and 1H3 or mouse IgG. After sacrifice, tumor nodules in lungs were counted. Data were pooled from three independent experiments (n = 9 or 10).

(B) BALB/c females were injected with B7x/4T1 in the mammary fat pad. Mice were i.p. treated with mAb 1H3. Tumor volumes were measured every 2 or 3 days after tumor injection. After mice were sacrificed, tumor weights were measured and tumor nodules on lungs were counted (n = 10). *$p < 0.05$.

(C) BALB/c mice were i.v. injected with CT26 cells expressing human B7x (hB7x/CT26) and then injected i.p. with mAb 1H3 or control IgG. After sacrificing the mice, tumor nodules in the lungs were counted (n = 9). Results were pooled from two independent experiments.

(D) Several human cancers were stained with 1H3 or IgG control using immunohistochemistry. Scale bars, 50 μm.

Effect of Anti-B7x Therapy on the Tumor Microenvironment

Vascular endothelial growth factor (VEGF) from tumor cells, stromal cells, and immune cells stimulates angiogenesis in the tumor microenvironment. This angiogenesis facilitates tumor growth and metastasis (Roda et al., 2012; Roland et al., 2009). We found that the VEGF concentration in tumor-bearing lungs from 1H3-treated mice was significantly lower than that of control mice (Figure 4I). The 1H3 treatment also lowered the concentration of transforming growth factor β (TGF-β) in tumor-bearing lungs (Figure 4J), one of the key cytokines responsible for suppressing antitumor immunity (Fridlender et al., 2009).

1H3 Kills Tumor Cells through ADCC

Antibodies can eliminate virus-infected cells or tumor cells via antibody-dependent cellular cytotoxicity (ADCC) (Clynes et al., 2000; Isitman et al., 2012; Kohrt et al., 2012), a mechanism requiring antibody, antigen-expressed target cells, and effector cells expressing Fc receptors. We examined whether 1H3 was able to kill tumor cells expressing B7x or tumor cells without expressing B7x through ADCC. We found that 1H3 induced 50% more target cells death compared to control IgG (Figures 5A and S4A).

1H3 Partially Blocks B7x-Mediated T Cell Coinhibition

It has been demonstrated that B7x inhibits T cell function in the presence of T cell receptor signaling in vitro (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003), but the receptor expressed on activated T cells is currently unknown. We next questioned whether 1H3 could inhibit B7x-mediated T cell coinhibition using a system modified from our previous studies (Zang et al., 2003). As expected, T cells proliferated vigorously when incubated with anti-CD3 and control immunoglobulin, with more than 73% of T cells dividing. When T cells were incubated with anti-CD3 and B7x-Ig, significantly fewer T cells proliferated, with about 41% dividing. The presence of 1H3 in the system significantly neutralized B7x-mediated T cell coinhibition, as 1H3 increased T cell proliferation to >61%. Furthermore, the Fab fragment of 1H3 had a similar neutralizing effect on B7x-induced T cell coinhibition (Figure 5B). These results reveal that 1H3 can partially block B7x-mediated T cell coinhibition. To assess whether 1H3 therapy depends on ADCC and/or functional neutralization

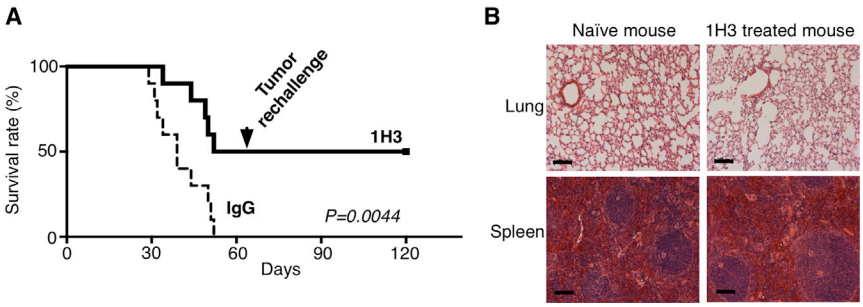

Figure 3. Effect of Anti-B7x Therapy on Survival and Tumor Rechallenge

(A) BALB/c mice were i.v. injected with B7x/CT26 at day 0 and then injected i.p. with 1H3 or control IgG. At day 60 postinjection, the surviving mice were i.v. rechallenged with B7x/CT26 (n = 10). *p < 0.05; **p < 0.01.

(B) At day 120, surviving mice were sacrificed and lung sections were used for H&E staining. Representative lung tissues from surviving and naive mice are shown. Scale bar, 100 μm.

in vivo, we compared the therapeutic efficacies of 1H3 and its Fab, which cannot mediate ADCC. Mice treated with Fab had significantly fewer lung tumor nodules than did mice treated with control IgG, but they had significantly more lung tumor nodules than did mice treated with 1H3 (Figure 5C). To confirm this effect, we validated the efficacy of 1H3 and Fab of 1H3 on the primary tumor model of B7x/4T1. 1H3 showed superior efficacy to that of 1H3 Fab (Figure S4B). Taken together, these results suggest that 1H3 inhibits tumor growth through the combination of ADCC and functional neutralization of B7x.

DISCUSSION

Here, we solved the structure of the functional IgV domain of B7x and developed a cancer immunotherapy targeting B7x. The B7 and CD28 families are very attractive therapeutic targets for human cancers. Compared to CTLA-4, PD-1, and PD-L1, which are targets for current clinical trials (Brahmer et al., 2012; Topalian et al., 2012), B7x has a very different expression pattern. In humans, CTLA-4, PD-1, and PD-L1 are not expressed on resting T cells, but they are induced after T cell activation and expressed on Tregs (Scandiuzzi et al., 2011). PD-1 and PD-L1 are induced on B cells, monocytes, and other immune cells after stimulation. By contrast, human B7x is hardly detected on immune cells even after stimulation (Lee et al., 2012). Neither CTLA-4 nor PD-1 are expressed on nonhematopoietic cells. PD-L1 is expressed on some normal tissues, whereas B7x is hardly detected in most human tissues (Choi et al., 2003). PD-L1 is expressed in some human cancers (Zang and Allison, 2007), whereas the expression of B7x in human cancers is more extensive, including cancers of the esophagus (Chen et al., 2011), lung (Sun et al., 2006), breast (Tringler et al., 2005), pancreas (Awadallah et al., 2008), kidney (Krambeck et al., 2006), gut (Jiang et al., 2010), skin (Quandt et al., 2011), ovary (Zang et al., 2010), and prostate (Zang et al., 2007). Therefore, the expression pattern of B7x suggests that this target is more cancer specific.

The overall organization of the human B7x IgV domain is similar to that of other B7 family members (e.g., a structural alignment with PD-L1 is shown in Figure 1C). In addition to the secondary structure topology, conserved features include the disulfide bond connecting strands B and F (formed by Cys56 and Cys130) and the tryptophan residue (Trp71) at the domain core. The receptor-binding interface described for other B7 family members is located on the front-sheet surface of their IgV domains (Lázár-Molnár et al., 2008; Schwartz et al., 2001; Stamper et al., 2001). Thus, it is likely that B7x engages its receptor on the equivalent surface. Furthermore, our observation of a large branched glycan of the back sheet of the B7x-IgV (linked to Asn112) would suggest that the back-sheet surface is unlikely to participate in receptor recognition. A glycosylation site at an equivalent position has been previously reported for the murine B7-H3 ectodomain (Vigdorovich et al., 2013).

We demonstrated that mAbs against B7x achieved significant therapeutic efficacy in mouse cancer models. A new study also demonstrates that the anti-B7x single-chain fragments variable (scFv) can delay OVCAR5 line growth in NSG mice (Dangaj et al., 2013). We and other groups have previously shown that activated T cells express an unidentified receptor for B7x (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003), and our recent work reveals that MDSCs also have a receptor for B7x (Abadi et al., 2013). These results suggest that the expression of the B7x receptors is broader than previously thought. Due to the lack of the identity of B7x receptors, we developed an in vivo functional screen for anti-B7x mAbs. We found that the expression of B7x on CT26 tumor cells significantly promoted tumor progression in vivo, which mirrored clinical observations, and was used to validate therapeutic mAbs. 1H3 and 12D11 were found to reduce more than 60% of tumor nodules in lungs, with development of a strong immunologic memory in the case of 1H3. Similar results were obtained with the 4T1 tumor model.

Characterization of 1H3's binding properties showed that in addition to recognizing murine B7x, it also bound strongly to the IgV domain of human B7x and was able to significantly inhibit tumor nodule formation of human B7x-expressing CT26 in lungs. These results suggest that humanized 1H3 may prove useful in the treatment of human cancers. To advance this strategy, we mapped the epitopes recognized by 1H3 on human B7x using a series of mutants whose design was guided by the structure of B7x-IgV. The clustering of the antibody-blocking mutants to the "top" of the molecule, in particular, residues Ile62 and Lys63 in the BC loop of the back sheet and residue Ser135 in the FG loop of the front sheet, defines the minimal footprint for the B7x:1H3 binding interface. The combination of 1H3 functional blocking data and the mutagenesis data suggests that the binding surface for the physiologically relevant B7x receptor partially overlaps with this surface. Our results are consistent with observations that IgV domains predominately interact with other IgV domains via strands and a loop on their front sheets like is seen in the interactions between CTLA-4 and PD-1 and their ligands (Figures S5A–S5D).

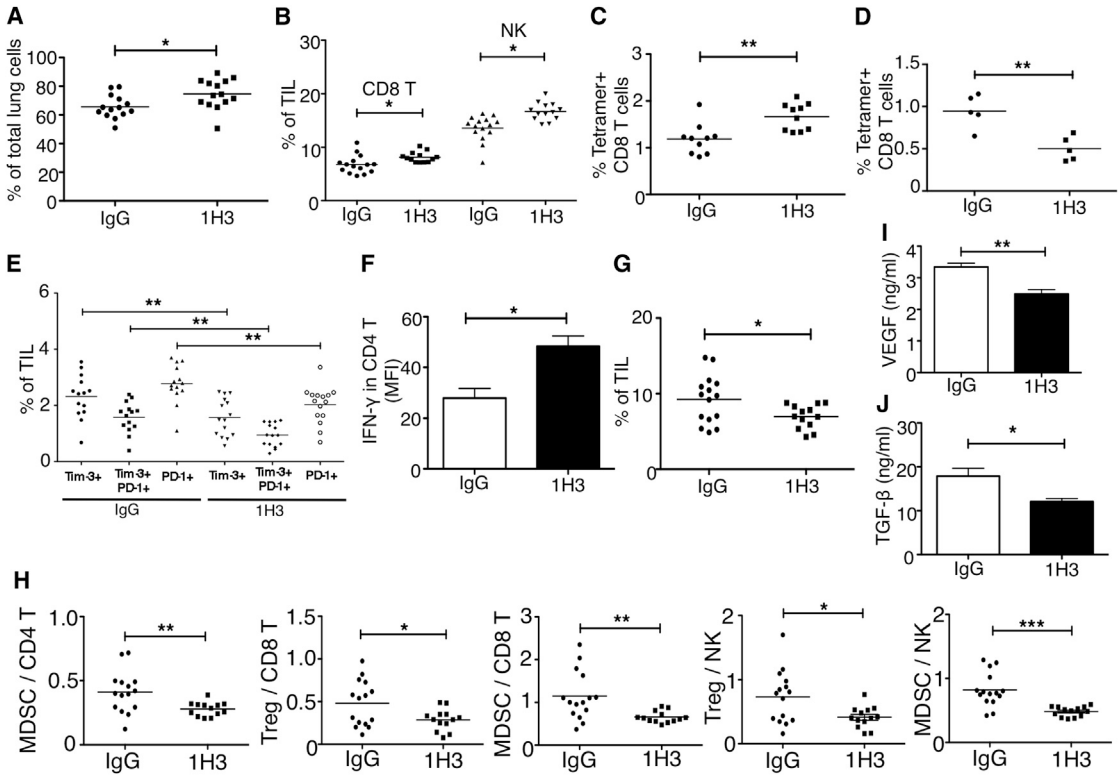

Figure 4. Anti-B7x Therapy Alters the Intratumor Balance of Antitumor Effector Immune Cells and Immunosuppressive Cells

(A–G) BALB/c mice were i.v. injected with B7x/CT26 and then treated with 1H3 or control mouse IgG. At day 17, single-cell suspensions from tumor-bearing lungs were analyzed by FACS for the percentage of infiltrated CD45$^+$ cells (A), the percentage of CD8 T cells and NK cells (B), the percentage of tumor antigen AH1 (SPSYVYHQF)-specific CD8 T cells (C), the percentage of CD4 T cells that were Tim-3+PD-1+, Tim-3+ alone and PD-1+ alone (E), and CD11b$^+$Ly6C$^+$ monocytic myeloid-derived suppressor cells (G). At day 17, single-cell suspensions from blood were analyzed by FACS for the percentage of tumor antigen AH1-specific CD8+ T cells were measured (D). Cell suspensions from tumor-bearing lungs were stimulated with 1× cell stimulation cocktail for 5 hr and stained with antibodies to CD3, CD4 and IFN-γ or isotype controls (F). Results are pooled from three independent experiments; *$p < 0.05$, **$p < 0.01$. Result in (D) is representative of data from two independent experiments.

(H) The ratios of Treg (Foxp3$^+$CD4$^+$) and MDSCs to CD8 T cells, CD4 T cells, and NK cells. These results are pooled from three independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(I) Total amount of VEGF from tumor-bearing lungs was measured using ELISA. **$p < 0.01$.

(J) Total amount of TGF-β from tumor-bearing lungs was measured using ELISA. Each group contained five mice. *$p < 0.05$.

It is surprising that 1H3 and 12D11 recognized similar epitopes in human B7x, because 12D11 showed no in vivo suppressive effect on human B7x-expressing CT26 cells. However, even though the difference was minor, 1H3 and 12D11 had at least one difference. Ile62's position in the IgV domain of human B7x seemed to play a significant role in the binding of B7x and anti-B7x mAbs. These results highlight the importance of our in vivo screening system to find therapeutic mAbs against B7x.

The studies presented here suggest that the principal mechanisms of the anti-B7x mAb in dampening tumor progression are the neutralization of the B7x-mediated coinhibition of T cells and the ADCC-mediated direct killing of tumor cells. We demonstrated that 1H3 killed B7x-expressing tumor cells in a dose-dependent manner and that both 1H3 and its Fab fragment partially recovered T cell proliferation suppressed by B7x-Ig fusion protein. In line with these results, the Fab treatment significantly reduced lung tumor nodules, but its therapeutic efficacy was not as great as full 1H3 mAb treatment. These results suggest that both neutralization and ADCC may take place in vivo during the 1H3 treatment. The 1H3 treatment strongly increased the infiltration of major types of antitumor immune cells such as CD8 T cells including tumor antigen-specific CD8 T cells, NK cells, and IFN-γ-producing CD4 T cells. On the other hand, the treatment markedly reduced infiltration of immunosuppressive MDSCs. As a consequence, the treatment shifted the tumor microenvironment to a favorable antitumor state with a significantly higher ratio of effector immune cells to the suppressive MDSCs and Tregs. Correspondingly, we found less VEGF and TGF-β in the tumor microenvironment after 1H3 treatment.

Therapies with mAbs against CTLA-4 and PD-1 target T cell coinhibitory receptors, whereas therapies with mAbs against PD-L1 and B7x target T cell coinhibitory ligands. The encouraging safety profile and antitumor activity from an anti-PD-L1 clinical trial (Brahmer et al., 2012), together with preliminary data from an anti-PD-1 trial that have suggested a correlation between tumor membrane PD-L1 expression and clinical response

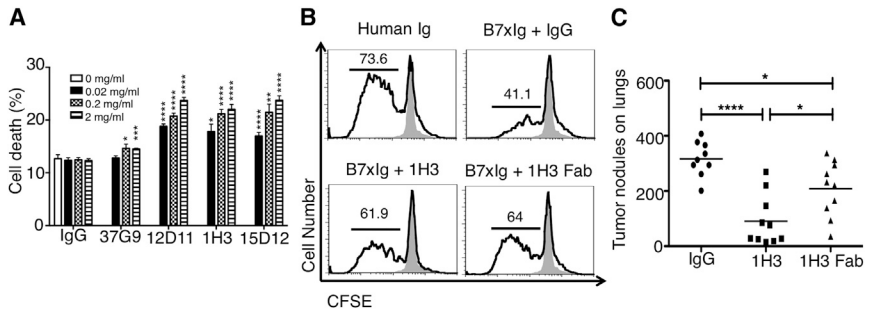

Figure 5. Antitumor Mechanisms of 1H3 Treatment

(A) mAbs against B7x killed CT26 tumor cells expressing B7x through antibody-dependent cellular cytotoxicity in vitro. Data are representative of two independent experiments in triplicates and shown as mean ± SE. $^{**}p < 0.01$; $^{****}p < 0.0001$.

(B) 1H3 partially neutralized B7x-mediated T cell coinhibition. CFSE-labeled T cells were incubated for four days with plate-bound anti-CD3 in the presence of control Ig, B7x-Ig and IgG (2 μg/ml), B7x-Ig and 1H3 (2 μg/ml), or B7x-Ig and 1H3 Fab (4 μg/ml). Representative FACS plots show CFSE dilution among live stimulated cells (solid line) compared to unstimulated control T cells (shaded area). Data are representative of two independent experiments.

(C) Comparison of therapeutic efficacies between 1H3 and its Fab. Data were pooled from two independent experiments (n = 9 or 10). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

to anti-PD-1 antibodies (Topalian et al., 2012), highlight the emerging significance of targeting coinhibitory B7 ligands. Further studies with humanized anti-B7x antibodies, either as monotherapy or in synergism with traditional therapies, should be pursued for the treatment of human cancers.

EXPERIMENTAL PROCEDURES

Protein Crystallization and Structure Determination

Purified human B7x IgV protein was concentrated to 10 mg/ml in HBS-E (10 mM HEPES [pH 7], 150 mM NaCl, 1 mM EDTA) and used to determine the initial crystallization conditions. Diffraction-quality crystals were obtained in 200 mM tripotassium citrate, 2.2 M ammonium sulfate, soaked in the well solution supplemented with 5-amino-2,4,6 triiodoisophthalic acid monohydrate (I3C) (Hampton Research) as a phasing reagent and 400 mM (final volume) lithium sulfate and flash-cooled in liquid nitrogen. Data sets at 1.075 Å and 1.54 Å wavelength were collected at the National Synchrotron Light Source beamline X29 (Upton, NY). Data were integrated using the iMosflm (Battye et al., 2011) with subsequent processing using the programs within the CCP4 software package (Winn et al., 2011). Single-wavelength anomalous dispersion (SAD) phasing was carried out using the anomalous iodine (I3C) signal from a data set collected at 1.54 Å, and the initial electron density maps were obtained using SHELXC/D/E (Sheldrick, 2008) and HKL2MAP. Following initial model building using ARP/wARP (Langer et al., 2008), the model was refined using REFMAC5 (Murshudov et al., 1997) with further manual building using Coot (Emsley et al., 2010).

Animals and Human Tissue Slides

BALB/c mice were purchased from the National Cancer Institute and The Jackson Laboratory. All mice were housed in a specific-pathogen-free facility. Human tumor tissue sections were obtained from IMEGENEX. All protocols were reviewed and approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee and Institutional Review Board.

Monoclonal Antibodies against B7x and Generation of Fab Fragment

Hybridoma cells from 37G9, 12D11, 1H3, and 15D12 clones were maintained in serum-free RPMI-1640 media (Wei et al., 2011). mAbs were purified on a Protein G column (Thermo Scientific). Fractions of eluted mAbs were measured and pooled for buffer exchange to PBS via dialysis. Fab fragment preparation was performed based on the manufacturer's protocol (Thermo Scientific).

Cell Lines and Tumors

CT26 and B7x/CT26 were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. 4T1 and B7x/4T1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 0.4 U/ml insulin as described previously (Abadi et al., 2013).

The 8- to 10-week-old mice were i.v. injected with 10^5 cells in 0.2 ml DMEM. For survival studies of CT26 and B7x/CT26, 10^5 B7x/CT26 cells were i.v. injected into mice and survival rate was examined. For survival studies of 1H3 treatment, 10^4 B7x/CT26 cells were i.v. injected into mice, and the same numbers of B7x/CT26 cells were injected for rechallenge. For the primary tumor experiment, 10^5 B7x/4T1 cells were injected subcutaneously into the mammary fat pad of female mice. Tumor nodules on lungs were measures as previously described (Abadi et al., 2013).

Anti-B7x Immunotherapy

A total of 200 μg of mAbs against B7x, 1H3 Fab, or normal mouse IgG were i.p. injected at days 1, 3, 7, 11, and 14. Each mouse received a total of 1 mg of antibody or Fab during B7x/CT26 experiments. At day 17, mice were sacrificed for counting tumor nodules in lungs. For hB7x/CT26 experiments, 200 μg of mAbs against B7x (1H3 and 12D11) or normal mouse IgG was i.p. injected at days 1, 2, 3, 5, 7, 9, 11, 13, and 15. Each mouse received a total of 1.8 mg antibody. At day 17, mice were sacrificed for counting tumor nodules in lungs. For the B7x/4T1 primary tumor model, 1 week after tumor injection, mice were grouped based on their tumor volume. A total of 300 μg of mAbs was i.p. injected at days 8, 11, 13, 15, and 18. Tumor volume was measured every 2 or 3 days after 7 days. At day 20, mice were sacrificed, breast tumors were excised and weighed, and tumor nodules on lungs were counted.

Human B7x Mutagenesis and Epitope Mapping

Ectodomain of hB7x (Gly21-Ala208) was cloned into a type I secretion ligation independent cloning vector we designed in house. Cloning into this vector fuses the target ectodomain to a nonnative transmembrane domain (TM domain from murine PD-L1) followed by the mCherry protein (as a cytosolic expression proxy). We have successfully used this vector to study expression, localization, and activity of several other members of the IgG superfamily. The wild-type hB7x type I secretion construct was transfected into human embryonic kidney 293S (HEK293S) cells. Cell-surface expression of B7x was confirmed by fluorescence-activated cell sorting (FACS) analysis using the B7x-specific mAb 1H3. Residues for site-directed mutagenesis were initially selected on the basis of our crystal structure of the hB7x-IgV domain to identify surface-exposed residues within the IgV domain. Surface-exposed residues clearly involved in a salt bridge were omitted from the set of targeted residues. Mutagenesis was performed using a standard PCR method using high-fidelity KOD polymerase. All of the successful mutants were sequence-verified and individually transfected into HEK293S cells using 24-well suspension plates. Two days posttransfection, the cells were counted and diluted to 1×10^6 cells/ml using 1× PBS with 2% BSA. A total of 0.2×10^6 cells were incubated with 2 μg of the 1H3 mAb for 1 hr at 4°C. Cells were subsequently pelleted by centrifugation at 500 × *g* and washed with PBS for a total of three times. Goat anti-mouse DyLight 488 secondary antibody (2 μg) was added to the cells, and they were incubated at 4°C for 45 min. Cells were washed, and antibody binding was assessed by FACS analysis on a BD Aria III. Flow cytometry data were gated for all mCherry-positive events (hB7x expression) and then subgated for anti-B7x binding (488 channel). To consider slight variation in total expression

of each mutant, the number of antibody-bound events was divided by the total number of mCherry-positive events. The experiment was performed in duplicate.

Statistics

Statistical analysis was performed with Prism software (GraphPad) using the unpaired Student's t test or the log-rank test (Mantel-Cox) for the survival study. p values < 0.05 were considered statistically significant.

Other detailed experimental procedures can be found in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

Final coordinates and structure factors were deposited in Protein Data Bank and are available under accession number 4GOS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.09.053.

AUTHOR CONTRIBUTIONS

H.J. performed experiments in immunotherapy. V.V. and U.A.R. determined the crystal structure. V.V. performed the affinity measurements. S.C.G performed epitope mapping. M.J. performed immunohistochemistry experiments. Y.M.A. helped with several experiments. J.S.L. established cell lines. L.S., K.C.O., J.M.C., R.Z., Y.Y., Y.M., and J.A.S. provided invaluable reagents and advice on experiments. H.J. and V.V. analyzed the data and wrote the manuscript. X.Z. and S.C.A. supervised the study and wrote the manuscript. All authors read and approved the manuscript.

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Review

CellPress

Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway

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The programmed death 1 (PD-1) receptor and its ligands programmed death ligand 1 (PD-L1) and PD-L2, members of the CD28 and B7 families, play critical roles in T cell coinhibition and exhaustion. Overexpression of PD-L1 and PD-1 on tumor cells and tumor-infiltrating lymphocytes, respectively, correlates with poor disease outcome in some human cancers. Monoclonal antibodies (mAbs) blockading the PD-1/PD-L1 pathway have been developed for cancer immunotherapy via enhancing T cell functions. Clinical trials with mAbs to PD-1 and PD-L1 have shown impressive response rates in patients, particularly for melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma (RCC), and bladder cancer. Further studies are needed to dissect the mechanisms of variable response rate, to identify biomarkers for clinical response, to develop small-molecule inhibitors, and to combine these treatments with other therapies.

Expression of PD-1 and its ligands

The PD-1 (CD279) (see Glossary) receptor can be detected at the cell surface of T cells during thymic development and in the periphery of several types of hematopoietic cell following T cell receptor (TCR) signaling and cytokine stimulation. PD-1 is expressed on $CD4^{-}CD8^{-}$ thymocytes and inducibly expressed on peripheral $CD4^{+}$ and $CD8^{+}$ T cells, B cells, monocytes, natural killer (NK) T cells, and some dendritic cells (DCs) [1,2]. Persistent expression of PD-1 on T cells induces T cell exhaustion [3]. Exhausted CD8 T cells lose their effector function, evidenced by their inability to secrete cytolytic molecules such as perforin and their failure to secrete proinflammatory cytokines, such as IL-2, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) [4,5].

$CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs), a highly immunosuppressive subset of $CD4^{+}$ T cells that is critical in maintaining tolerance and attenuating immune responses, express cell-surface PD-1, which contributes to their development, maintenance, and functional response [6]. Ligand binding to the PD-1 receptor on Tregs in the presence of CD3 and transforming growth factor beta (TGF-β) leads to an increase in the *de novo* conversion of naive $CD4^{+}$ T cells to Tregs. This induction generates heightened suppressive function and maintenance of Foxp3 expression through inhibition of Akt–mammalian target of rapamycin (mTOR) signaling and increased phosphatase and tensin homolog (PTEN) activity [7,8]. This indicates that PD-1 pathway stimulation results not only in a reduction in effector T cell function, but also an increase in immunosuppressive Treg function. This allows proper control of immune homeostasis and creates a high threshold for T cell activation.

Although PD-1 has best been characterized in T cells, its function in other cell subsets have also become apparent. The regulation of PD-1 expression is tightly controlled during B cell differentiation. Levels are undetectable in pro-B cells, an early precursor in B cell development, and increase as B cell differentiation [9]. Additionally, surface levels of PD-1 can be greatly enhanced in mature B cells following stimulation with Toll-like receptor 9 (TLR9) agonists. Blockade of PD-1 on B cells has been shown to increase antigen-specific antibody responses,

Glossary

Cancer immunotherapy: treatments that use the host immune system to inhibit cancer.
Monoclonal antibody (mAb): antibodies generated by immune cells derived from a single parent cell.
Programmed death 1 (PD-1): a 288-amino acid cell-surface molecule, encoded in humans by the *PDCD1* gene, that functions to negatively regulate immune responses.
Programmed death ligand 1 (PD-L1): a 40-kDa type 1 transmembrane protein, encoded in humans by the *CD274* gene, that suppresses the immune system in cancer, pregnancy, tissue allografts, and autoimmune diseases.
T cell: a type of lymphocyte that has an important role in cell-mediated immunity, distinguished by its T cell receptor on the cell surface; referred to as T cells because they mature in the thymus.
T cell coinhibition: a signal required for inhibition of activated T cells in the presence of T cell receptor signal.

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suggesting that PD-1 plays a role in inhibiting B cell clonal responses [10].

PD-1 has two binding ligands, PD-L1 (B7-H1, CD274) [11,12] and PD-L2 (B7-DC, CD273) [13,14], with PD-L1 being the most prominent in regulation. PD-L1 is inducibly expressed on both hematopoietic cells and non-hematopoietic cells following cell-specific stimulation. Cytokines such as IFN-γ and TNF-α upregulate the expression of PD-L1 on T cells, B cells, endothelial cells, and epithelial cells, furthering its role in the maintenance of peripheral tolerance [1]. Data also link genetic changes seen in cancer cells to the induction of PD-L1, although this can vary by cancer type. PTEN dysfunction in human glioma cells induces Akt activation and subsequently PD-L1 expression, while human melanoma cells show no association between PTEN or Akt and PD-L1 induction [15,16]. Recent data show that PD-L1 binds to B7-1 (CD80) in addition to PD-1 [17]. While PD-L1 expression is induced on a wide array of both hematopoietic and non-hematopoietic cells, PD-L2 expression is restricted to inducible expression on DCs, macrophages, mast cells, and some B cells in response to IL-4 and IFN. The affinity of PD-L2 for PD-1 is three times greater than that of PD-L1, which indicates competition between the two ligands. Recent data confirm a second cognate receptor for PD-L2, repulsive guidance molecule B (RGMb) [18]. Despite recent research efforts surrounding PD-L2, little is known regarding the transcriptional regulation of the ligand.

Structures of PD-1 and its ligands

Structurally, PD-1 is a type I transmembrane receptor and belongs to the Ig superfamily (IgSF). Although it is functionally related to the costimulatory/coinhibitory receptors CD28, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and inducible T cell costimulator (ICOS), PD-1 has important structural and functional differences. Other receptors in the CD28 family are disulfide-linked dimers; however, structural and cell-surface studies demonstrated that PD-1 is a monomeric glycoprotein [19]. The crystal structure of the extracellular region of mouse PD-1 shows the presence of a typical Ig variable domain (IgV) comprising front sheets (A'GFCC'C") and back sheets (ABED) (Figure 1) stabilized by a disulfide bond linking the F and B strands [19]. This IgV domain is linked to transmembrane and cytoplasmic domains through a 20-amino acid stalk region. In contrast to other CD28 family receptors, the absence of an extracellular cysteine residue in the stalk region prevents PD-1 from covalent dimer formation.

Human and mouse PD-1 share around 60% overall identity at the protein level, which increases to 75% for the residues forming the IgV domain. It is unsurprising, therefore, that crystallographic [Protein Data Bank (PDB) code 3RRQ] and NMR structures [20] show a high degree of similarity between mouse and human PD-1. Overlay of the crystal structures of mouse and human PD-1 shows very similar arrangements (Figure 1). One notable difference between human and mouse PD-1 is the lack of the C" strand at the edge of the front GFCC' sheet in human PD-1, as shown by the NMR data [20]. This region instead presents as a highly flexible loop, consistent with the poor

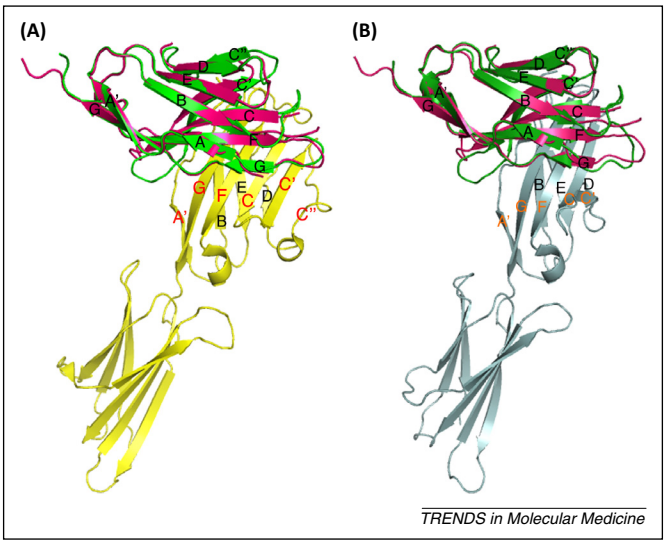

Figure 1. Crystal structures of programmed death 1 (PD-1) receptor/programmed death ligand 1 (PD-L1) and PD-1/PD-L2 complexes. **(A)** Overlay of the crystal structures of human PD-1 [Protein Data Bank (PDB) code 3RRQ] and the mouse PD-1/human PD-L1 complex (3BIK). **(B)** Overlay of human PD-1 with the mouse PD-1/PD-L2 complex (3BP5). Pink, human PD-1; green, mouse PD-1; yellow, human PD-L1; grey, mouse PD-L2.

electron density observed for that region in the crystallographic dataset, indicating a disordered arrangement (PBD code 3RRQ).

Another unique structural feature of PD-1 is that it lacks a consensus complementarity-determining region 3 (CDR3)-like conserved ligand-binding motif. The ligand-binding site comprises a hydrophobic patch on the front face contributed by multiple residues from several strands [19]. Crystal structures available for complexes of mouse PD-1 and human PD-L1 [21] and mouse PD-1 with mouse PD-L2 [22] show similar overall molecular architecture for these inhibitory complexes. Both PD-1 and its ligands interact with their respective surface residues distributed over their front beta sheets (front-to-front binding). By contrast, the FG loop of PD-1, which corresponds to the CDR3 variable region of the Ig structure, makes little or no contact with PD-L1 or PD-L2 (Figure 1). The crystal structures of the PD-1/PD-L complexes reveal that PD-1 binds its ligands with 1:1 stoichiometry and forms monomeric complexes. This indicates a distinct ligand-binding mode and signaling mechanism that differs from other coinhibitory receptor/ligand interactions such as CTLA-4/B7, where oligomerization plays an important role in signaling. Although crystal structures for the human PD-1/PD-L1 and PD-1/PD-L2 complexes remain to be solved, the overall similarity of the mouse and human PD-1 structures suggests that mouse and human PD-1 are likely to form similar complexes. Overlay of human PD-1 with the mouse complexes shows that human PD-1 may bind to its ligands in the same way as mouse PD-1; however, a recent study using NMR with binding data and mathematical modeling suggests that PD-1 may be engaged by its two ligands differently [20].

Importantly, the available crystal structures of PD-1 and the PD-1/PD-L1 and PD-1/PD-L2 complexes allow not only mapping of the ligand-binding sites and mAb blocking epitopes, but also the design of small-molecule inhibitors.

Table 1. Prognostic significance of PD-1 expression in human tumor-infiltrating lymphocytes

Tumor	Clinical correlation	Refs
Breast	High tumor-infiltrating PD-1$^+$ cell counts decreased patient survival	[54]
Breast	PD-1$^+$ TILs associated with tumor size, grade, LN status, and worse overall survival	[55]
Prostate	CD8$^+$ TILs expressed high levels of PD-1 and had restricted TCR Vbeta gene usage	[56]
Thyroid	PD-1$^+$ T cells in LNs were indicative of recurrent disease and correlated with Treg frequency	[57]
Melanoma	PD-1$^+$ TILs expressed CTLA-4, displayed an exhausted phenotype, and were functionally impaired compared with PD-1$^-$ TILs	[58]
Melanoma	PD-1 expression on CD4$^+$/CD8$^+$ T cells was found in primary tumor, with greater expression in distant metastases	[59]
Ovarian	CD8$^+$PD-1$^+$ T cells were impaired in IFN-γ/TNF-α secretion compared with CD8$^+$PD-1$^-$ T cells	[60]
RCC	Presence of PD-1$^+$ intratumoral immune cells associated with advanced stage and significant risk for cancer-specific death compared with PD-1$^-$ patients	[61]
NSCLC	CD8$^+$ TILs increased PD-1 expression resulting in reduced cytokine production and capacity to proliferate	[62]
HCC	CD8$^+$PD-1$^+$ TILs predicted disease progression and tumor recurrence	[63]

Prognostic relevance of PD-1 and its ligands in human malignancies

Persistent expression of PD-1 by T cells is highly indicative of an exhausted phenotype, noted by a decrease in effector function [4,5]. This phenotype has been observed in various types of tumor-infiltrating lymphocyte (TIL) and linked to poor prognosis and tumor recurrence, highlighting PD-1 as an important molecule in regulating antitumor activity (Table 1). Similar to PD-1, PD-L1 and PD-L2 also possess prognostic capacities in some human malignancies (Table 2). Some clinical studies associate high expression of PD-L1 in tumors to tumor size, lymph node (LN) involvement, grade, and overall survival, while PD-L2 has generally been tied only to a trend in decreased survival that is not of statistical significance (Table 2). PD-L1 generally has a much broader expression pattern compared with PD-L2. This indicates that the regulation of PD-L2 depends much more on environmental stimuli than that of PD-L1. Data from these studies provide a solid rationale for investigating the immunological mechanisms behind the clinical associations. The poor prognosis indicated by the expression of PD-1 on TILs and of PD-L1/2 on tumor cells supports the targeting of the pathway therapeutically.

Mechanisms of anti-PD-1 and anti-PD-L1 immunotherapy

Appreciating the consequences of the upregulation of the PD-1/PD-L1/2 axis aids our progress in manipulating an immunosuppressive cancer microenvironment. The cytoplasmic tail of PD-1 contains two signaling motifs. One is an immunoreceptor tyrosine-based inhibitory motif (ITIM) and the other is an immunoreceptor tyrosine-based switch motif (ITSM). Binding of PD-L1 or PD-L2 to PD-1 on activated T cells, along with TCR signaling, leads to phosphorylation of the cytoplasmic domain tyrosines and recruitment of a Src homology 2-containing tyrosine phosphatase (SHP-2) to the ITSM. Consequently, SHP-2 dephosphorylates TCR-associated CD-3ζ and zeta chain-associated protein kinase 70 (ZAP70), resulting in inhibition of downstream signaling including blocking phosphoinositide 3-kinase (PI3K) and Akt activity, disrupting glucose metabolism and IL-2 secretion [5,23].

mAbs have been developed for cancer immunotherapy by enhancing T cell function via blockade of the binding between PD-1 and PD-L1 or PD-L2 (Figure 2). Many of these studies have shown that blockade of PD-1 alone or PD-L1 leads to an increase in T cells and IFN-γ at the tumor site [24], along with decreases in the percentages of the highly immunosuppressive myeloid-derived suppressor cell (MDSC) population [25]. Increase in the effector-to-suppressor cell ratio usually supports an antitumor microenvironment. These results demonstrate that the neutralization of PD-1, PD-L1, or PD-L2 can be effective in

Table 2. Prognostic significance and pathological associations of PD-L1 and PD-L2 on human tumor cells

Tumor	Clinical correlation	Refs
Colon	PD-L1 expression was associated with TNM stage and predicted prognosis	[64]
Cervical	PD-L1 was expressed in only a minority of samples and influences patient survival	[65]
Pancreatic	PD-L1-positive patients had poorer prognosis than PD-L1-negative patients and PD-L1 was inversely correlated with CD8$^+$ TILs; PD-L2 showed no significant correlation with patient survival	[66]
Breast	PD-L1 expression was correlated with tumor size, grade, LN status, and significantly worse overall survival	[67]
Ovarian	PD-L1 expression on monocytes in ascites and blood from patients with malignant ovarian carcinoma was greater than in those with borderline/benign disease; PD-L1 expression led to poorer prognosis and was inversely correlated with intraepithelial CD8$^+$ T cells, while PD-L2 showed poorer prognosis but not a significant difference	[68,69]
RCC	Soluble PD-L1 was associated with larger tumors, worse stage, grade, and necrosis, and increased risk of death; PD-L1 was associated with poor prognosis	[70,71]
HCC	PD-L1 expression on hematoma cells enriched apoptotic CD8$^+$ T cells; greater expression of PD-L1 was associated with significantly poorer prognosis and was an independent predictor for recurrence, while PD-L2 expression correlated with poorer survival but not recurrence	[63,72]
NSCLC	PD-L1 was associated with EGFR[a] mutations and was a negative prognostic factor	[73]
Melanoma	Greater PD-L1 expression correlated with significantly lower overall survival and vertical growth of primary tumors; PD-L1 marks a subset of melanomas with shorter overall patient survival	[74,75]
Esophageal	PD-L1 and PD-L2 expression led to significantly poorer prognosis while only PD-L2 expression was inversely correlated with CD8$^+$ TILs	[76]

[a]Epidermal growth factor receptor.

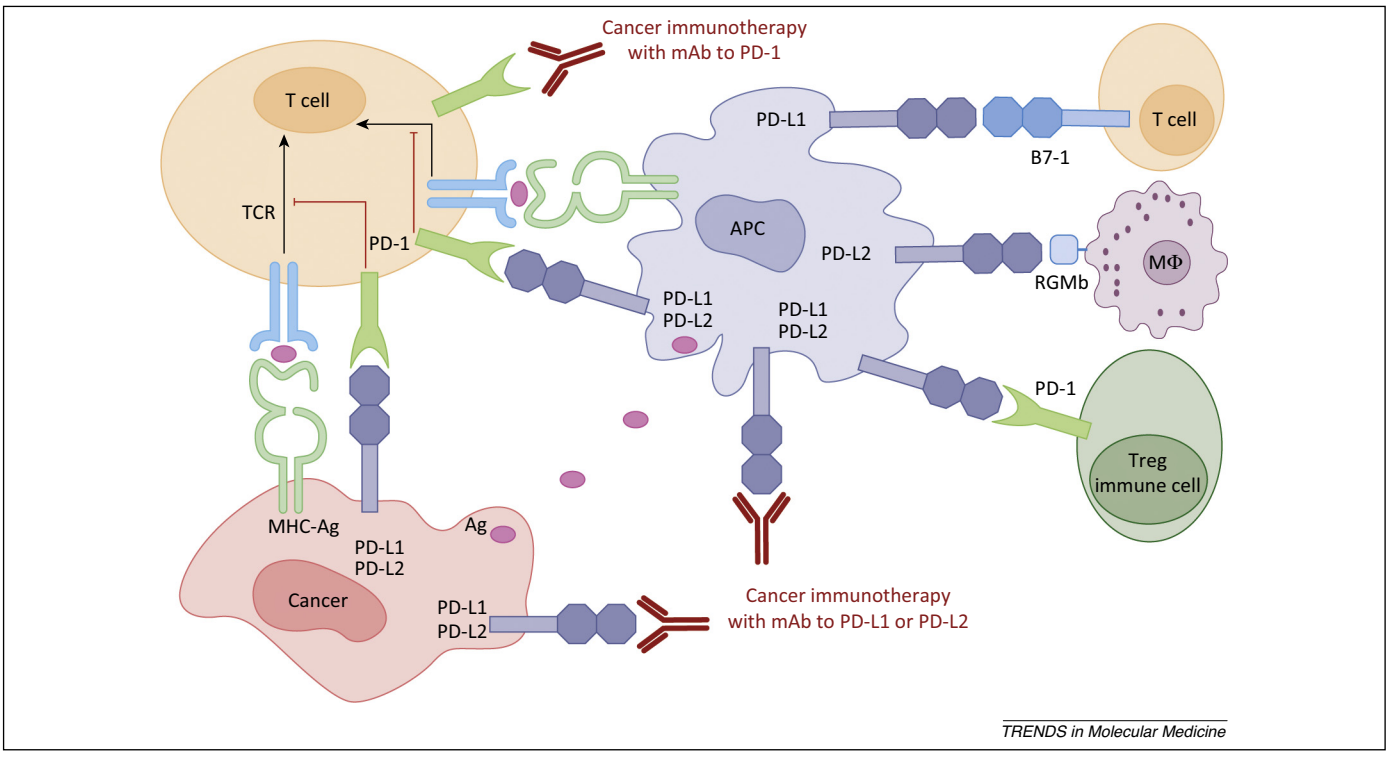

Figure 2. Human cancer immunotherapy with anti-programmed death 1 (PD-1) receptor and anti-programmed death ligand 1 (PD-L1)/L2 antibodies. Antigen-presenting cells (APCs) take up antigen (Ag) released from cancer cells and present it to T cells. Cancer cells can also present Ag to activated T cells in the context of the MHC. On T cell activation, PD-1 receptors are expressed on T cells and inhibit immune responses by engagement of PD-L1 and PD-L2 on APCs and PD-L1 on cancer cells. Therefore, monoclonal antibody (mAb)-mediated specific blockade of the PD-1/PD-L1/PD-L2 pathway can enhance antitumor immunity. In addition to binding to PD-1, PD-L1 and PD-L2 also bind B7-1 and repulsive guidance molecule B, respectively. In addition to T cells and APCs, PD-1 and PD-L1 can be induced on other immune cells.

controlling tumor growth by changing the dynamic of the tumor microenvironment.

Additional approaches generating synergy are the blockade of PD-1 or PD-L1 in combination with other therapeutic agents. Simultaneous blockade of both PD-1 and CTLA-4 leads to expansion of TIL populations while reducing the number of MDSCs within the tumor, leading to tumor regression and significant increases in IFN-γ and TNF-α in $CD8^+$ T cells [26]. Furthermore, chemotherapy and radiotherapy are being studied in combination with the blockade of the PD-1/PD-L1 pathway [27,28]. Together these results set the stage for an optimistic clinical outlook.

Various biological inhibitors of PD-1 and PD-L1 have been developed and are currently being tested in clinical trials with cancer patients (Table 3). These inhibitors include mAbs to PD-1 and PD-L1 as well as PD-L2 fusion protein.

Clinical trials of mAbs to PD-1

Pidilizumab (CT-011) was the first mAb to PD-1 to reach clinical trials [29] (Table 4). It was initially identified as a mAb binding to the B lymphoblastoid cell line that stimulated murine lymphocytes and showed antitumor activity in mice [30]. It stimulated human peripheral blood lymphocytes and enhanced cytotoxicity toward human tumor cell lines. The first Phase I trial with pidilizumab recruited patients with hematologic malignancies, including acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), Hodgkin's lymphoma (HL), and multiple myeloma (MM) [29]. Dose levels ranged from 0.2 to 6 mg/kg. A maximum tolerated dose (MTD) was not reached and the drug was well tolerated. Of the 17 patients enrolled in the study, one patient experienced a complete response, four had stable disease, and one had a mixed response, amounting to a 33% clinical benefit rate. Durable responses of greater than 60 weeks were

Table 3. Biological agents targeting PD-1 or PD-L1 in cancer clinical trials

Biological agent	Class	Target	Company	Refs
CT-011 (pidilizumab)	Humanized IgG1	PD-1	CureTech	[29]
MK-3475 (lambrolizumab, pembrolizumab)	Humanized IgG4	PD-1	Merck	[33]
BMS-936558 (nivolumab)	Human IgG4	PD-1	Bristol–Meyers Squibb	[37]
AMP-224	PD-L2 IgG2a fusion protein	PD-1	Amplimmune/GlaxoSmithKline	[52]
BMS-936559	Human IgG4	PD-L1	Bristol–Meyers Squibb	[44]
MEDI4736	Humanized IgG	PD-L1	MedImmune	[45]
MPDL3280A	Human IgG	PD-L1	Roche	[49]
MSB0010718C	Human IgG1	PD-L1	Merck	[51]

Table 4. Summary of reported clinical trials of mAbs that target PD-1 or PD-L1 as monotherapy, or in combination therapy with other agents, in human cancer

Antibody	Dose	Phase	Cancer	NCT[a] Number	Refs
Monotherapy					
Pidilizumab	0.2–6 mg/kg	I	AML, CLL, NHL, HL, MM	N/A	[29]
Pidilizumab	1.5 or 6 mg/kg	II	Malignant melanoma	NCT01435369	[77]
Pembrolizumab	1–10 mg/kg	I	Advanced solid tumors	NCT01295827	[33,34,36,78]
Nivolumab	0.3–10 mg/kg	I	Advanced solid tumors	NCT00441337	[39]
Nivolumab	1–10 mg/kg	I	Advanced solid tumors	NCT00730639	[40]
Nivolumab	1–20 mg/kg	I	Advanced solid tumors	N/A	[79]
Nivolumab	0.3–10 mg/kg	II	RCC	NCT01354431	[80]
Nivolumab	1 or 3 mg/kg	II	Platinum-resistant ovarian cancer	N/A	[81]
BMS-936559	0.3–10 mg/kg	I	Advanced solid tumors	NCT00729664	[44]
MPDL3280A	1–20 mg/kg	I	Advanced solid tumors and disease-specific cohorts	NCT01375842	[49,50,82–84]
MEDI4736	0.1–15 mg/kg	I	Advanced solid tumors	NCT01693562	[46]
MSB0010718C	1–20 mg/kg	I	Refractory malignancies	NCT01772004	[51]
Combination therapy					
Pidilizumab after ASCT	1.5 mg/kg	II	NHL	NCT00532259	[32]
Pidilizumab + rituximab	3 mg/kg	II	Follicular lymphoma	NCT00904722	[31]
Nivolumab + ipilimumab	1 mg/kg + 3 mg/kg	I	Malignant melanoma	NCT01024231	[43]
Nivolumab + platinum and nivolumab + ipilimumab	10 mg/kg and 1 mg/kg + 3 mg/kg	I	NSCLC	NCT01454102	[85,86]
Nivolumab + ipilimumab	3 mg/kg + 1 mg/kg	I	RCC	NCT01472081	[87]
Nivolumab + multipeptide vaccine	1–10 mg/kg	I/II	Malignant melanoma	NCT01176461	[88]

[a]National Clinical Trial.

noted. This was followed by two Phase II clinical trials [31,32]. Patients with diffuse large B cell lymphoma (DLBCL) or primary mediastinal B cell lymphoma (PMBCL) who underwent autologous hematopoietic stem cell transplantation (ASCT) and who had chemosensitive disease were treated with pidilizumab at 1.5 mg/kg every 42 days for three cycles starting 30–90 days post-transplantation [32]. The study enrolled 72 patients. Sixteen-month progression-free survival (PFS) for eligible patients was 72%, meeting the primary end point of the study. Intention-to-treat analysis revealed a 16-month PFS of 68%. The overall response rate for patients with measurable disease after ASCT was 51%. The most common grade 3 or 4 toxicities included neutropenia and thrombocytopenia. Correlative studies of select lymphocyte subsets revealed an increase in the number of activated $CD25^+PD\text{-}L1^+CD4^+$ T cells, $PD\text{-}L1^+PD\text{-}L2^+CD14^+$ monocytes, and circulating peripheral and central memory CD8 T cells as well as central memory CD4 T cells. These results suggest that pidilizumab may reverse PD-1-mediated inhibition of T cell survival and proliferation.

The second Phase II study with pidilizumab was a combined treatment with rituximab for follicular lymphoma [31]. Patients with rituximab-sensitive disease were treated with four doses of pidilizumab at 3 mg/kg every 4 weeks, with the option to continue therapy if they showed a response or stable disease. The study enrolled 32 patients. Patients were fairly well distributed across the three risk groups of the Follicular Lymphoma International Prognostic Index (FLIPI) 1 and 2. The objective response rate was 66%, which met the study end point of greater than 60% compared with a historical response rate of 40% with rituximab alone. The complete response rate was 52%. Responses were durable, with a median PFS of 18.8 months. FLIPI 1 or 2 score was not associated with response rate. The regimen was well tolerated, with no grade 3 or 4 adverse events. PD-L1 expression was significantly higher on $CD4^+$, $CD8^+$, and $CD14^+$ peripheral blood cells from responding patients but was not associated with PFS. Gene expression data suggested that intrinsic antilymphoma immunity might be predictive of a response to pidilizumab. Pidilizumab continues to be evaluated in various clinical trials, including solid tumors and hematologic malignancies, both as a single agent and in combination with other regimens including cellular therapies and cancer vaccines.

Pembrolizumab (MK-3475; previously known as lambrolizumab) (Table 4) is a humanized IgG4 PD-1-blocking mAb [33,34]. It is also the first mAb targeting PD-1 that has been granted accelerated FDA approval. A very high-affinity mouse antihuman PD-1 antibody was developed, the variable region of which was grafted to human IgG4 with a stabilizing S228P Fc mutation. The IgG4 subtype does not engage Fc receptors or activate complement, thus avoiding cytotoxic activity against T cells. Pembrolizumab was studied in a Phase I trial in patients with advanced solid tumors [33]. The dose range was 1–10 mg/kg and a MTD was not identified. In the nine patients enrolled in the study, no grade 3 or 4 toxicities were noted. One patient with melanoma experienced a partial response, with an additional three patients experiencing stable disease. Pembrolizumab activity and safety in melanoma was further explored by recruiting an expansion cohort at the 10 mg/kg dose level [34]. Doses ranged from 2 mg/kg every 3 weeks to 10 mg/kg every 2 weeks. Patients with advanced melanoma, including patients who had received prior treatment with ipilimumab, an FDA-approved mAb to CTLA-4, were allowed in this study. Results from 135 treated patients were reported. The response rate across all

dose cohorts was 38%, with the highest response rate observed in the cohort given 10 mg/kg every 2 weeks (52%). Responses were durable and overall PFS was longer than 7 months. Median overall survival (OS) was not reached. Treatment-related grade 3 or 4 adverse events were reported in 13% of patients. The highest incidence of treatment-related adverse events was in the group given 10 mg/kg every 2 weeks. Endocrine toxicities included hypothyroidism in 8.1% of patients, with one case being grade 3 or 4, grade 3 hyperthyroidism, and grade 2 adrenal insufficiency. Correlative studies on available tumor biopsies showed that regressing lesions were densely infiltrated with cytotoxic T lymphocytes (CTLs), which was consistent with the mechanism of action of the drug. PD-L1 expression by tumor cells was significantly associated with PFS and response rate but not with OS [35]. It is important to note here that, despite a very low cut-off for PD-L1 positivity (1% of stained cells), antitumor activity was noted in tumors with low PD-L1 expression. These results cast doubt on the utility of PD-L1 expression as a biomarker and suggest that the mechanism of action of PD-1-targeting antibodies remains to be fully elucidated.

To explore the efficacy of pembrolizumab in malignant melanoma patients who had progressed after ipilimumab or treatment with a BRAF or mitogen-activated protein kinase kinase (MEK) inhibitor, or both, an open-label, randomized, multicenter expansion cohort of the KEYNOTE-001 trial was performed [36]. The trial randomized 173 patients to receive pembrolizumab at 2 mg/kg or 10 mg/kg every 3 weeks. The overall response rate was 26% in both groups. Survival at 1 year was similar in the two treatment groups (58% and 63%). Pembrolizumab was well tolerated, with drug-related grade 3 or 4 adverse events reported in 12% of patients in both arms. Six patients (3%) discontinued treatment due to adverse events. Three patients experienced immune-related adverse events and were managed with dose interruption and corticosteroid treatment. Results from the KEYNOTE-001 trial served as the basis for the accelerated FDA approval of pembrolizumab for the treatment of patients with advanced or unresectable melanoma that progressed after therapy with ipilimumab or after therapy with a BRAF inhibitor in tumors carrying the BRAF V600 mutation. Of note in this trial is that the two dose levels exhibited similar antitumor activity and toxicity profiles. Pembrolizumab continues to be evaluated in Phase I trials in advanced solid tumors, head and neck cancers, and hematologic malignancies and in combination with lenalidomide and dexamethasone in relapsed/refractory MM, as well as in Phase II and III trials in microsatellite unstable tumors, in combination with pazopanib in renal cell cancer, and compared with docetaxel in NSCLC.

Nivolumab (MDX-1106, BMS-936558, ONO-4538) (Table 4) is a fully human IgG4 mAb to PD-1. In a humanized *in vitro* model of melanoma, addition of nivolumab to human vaccine-induced $CD8^+$ T cells specific to melanoma antigens allowed the expansion of these lymphocytes [37]. In another study of an *ex vivo* melanoma model, addition of the same antibody led to the unmasking of CTL inhibition by Tregs and stimulated their proliferation [38]. Nivolumab was first studied clinically in a Phase I trial in patients with advanced solid tumors. Thirty-nine patients with metastatic melanoma, colorectal cancer (CRC), castrate-resistant prostate cancer (CRPC), NSCLC, or RCC were enrolled [39]. Initial treatment entailed a single infusion of nivolumab in dose-escalating, six-patient cohorts at 0.3, 1, 3, or 10 mg/kg. This was followed by a 15-patient expansion cohort at the 10 mg/kg level. Patients who had shown clinical benefit at 3 months were eligible for repeated therapy. MTD was not reached. One durable complete response of greater than 21 months was noted in a patient with CRC and two partial responses were noted in a patient with melanoma and another with RCC. Two additional responses were noted in one melanoma and one NSCLC patient, but did not meet partial response criteria. Tumor biopsies were available from a few patients and PD-L1 expression appeared to correlate with response to therapy. A cut-off of 5% of tumor cells exhibiting membranous PD-L1 staining was used to define positivity for PD-L1 expression. Nivolumab was well tolerated, with one patient experiencing grade 3 inflammatory colitis treated with infliximab and steroids.

Nivolumab was further studied in another large Phase I study [40]. Patients were treated with escalating doses ranging from 1 to 10 mg/kg. Analysis of 304 patients, including expansion cohorts, revealed that nivolumab was well tolerated, with no MTD reached. Objective response rates for melanoma, NSCLC, and RCC were 31%, 16%, and 29%, respectively. No responses were noted in patients with CRC or CRPC. Responses were durable and lasted over 1 year. Updated results indicate that responses are durable in patients with NSCLC, with OS rates of 12–45% at 2 years [41]. Similarly, 2- and 3-year OS rates in patients with advanced melanoma were 48% and 41%, respectively [42]. Examination of PD-L1 expression revealed that responses were noted only in PD-L1-positive tumors (36%). Again, a cut-off of 5% of tumor cells staining positive for membranous PD-L1 expression by immunohistochemistry was used to define positivity. Grade 3 or 4 adverse events were noted in 15% of patients and 6% discontinued treatment. Adverse events with potentially immune-related etiology occurred in 45% of patients, being grade 3 or 4 in 6% of these. Three deaths due to pneumonitis were reported. Other studies of nivolumab have reported similar results, as detailed in Table 4.

Nivolumab was also studied in combination with ipilimumab in a Phase I trial [43]. The trial included a combined-regimen arm that enrolled 53 patients and a sequenced-regimen arm that enrolled 33. The MTD was determined to be at the 1 mg/kg nivolumab and 3 mg/kg ipilimumab dose level (cohort 2) and at 3 mg/kg for nivolumab and 1 mg/kg for ipilimumab (cohort 2a) in the combined regimen. Response rates were 40% in the combined regimen group and up to 53% in the MTD cohort. Complete responses were noted as well as 80% tumor reduction in 16 patients. Responses were durable, ranging from 6.1 to 72.1 weeks at the time of analysis. Treatment-related grade 3 or 4 adverse events were observed in 53% of patients and serious adverse events were reported in 49% of patients in the combined-regimen group; 21% of patients discontinued therapy due to adverse events. Adverse events were managed with immune suppressants, some requiring infliximab or mycophenolate mofetil. No

treatment-related deaths were reported. Regarding PD-L1 expression by tumor cells, 21 of 56 patients (38%) were positive for PD-L1 on immunohistochemistry, but this did not correlate with response. This study utilized a different anti-PD-L1 clone and a cut-off of 5% of tumor cells expressing PD-L1 was used to define positivity. Responses were noted in PD-L1-negative tumors.

Ongoing trials of nivolumab include Phase I trials in hematologic malignancies, hepatocellular carcinoma (HCC), and malignant melanoma, in combination with sunitnib, pazopanib, or ipilimumab in RCC, with ipilimumab in advanced solid tumors, in combination with IL-21 in advanced solid tumors, with a multipeptide vaccine in malignant melanoma, with lirilumab (anti-KIR; BMS-986015) in advanced solid tumors, and with dasatinib in relapsed chronic myelogenous leukemia (CML). Ongoing Phase II and Phase III trials with nivolumab are being conducted with or without ipilimumab versus bevacizumab in glioblastoma and with ipilimumab in malignant melanoma, compared with chemotherapy in NSCLC and malignant melanoma, and compared with everolimus in RCC.

Clinical trials of mAbs to PD-L1

BMS-936559 is a fully human monoclonal IgG4 antibody that blocks PD-L1 [44] (Table 4). This blockade was shown to augment T cell proliferation in response to allogeneic dendritic cells in a mixed lymphocyte reaction, as well as antigen-specific T cell responses to cytomegalovirus (CMV) antigen and antitumor peptide responses in subjects treated with melanoma antigen peptide vaccines. BMS-936559 can reverse *in vitro* Treg-mediated suppression. Use of BMS-936559 in clinical trials was supported by the ability of anti-PD-L1 antibodies to inhibit tumor growth in murine syngeneic tumor models and long-lived antitumor immunity was observed. BMS-936559 did not induce antibody-dependent cytotoxicity or complement-dependent cytotoxicity in PD-L1-positive cells. BMS-936559 was studied in a Phase I trial [44]. Patients with various solid tumors were enrolled, with disease-specific expansion cohorts enrolled in parallel and treated at the 10 mg/kg dose level. MTD was not reached and the discontinuation rate due to adverse events was 11%, 6% of which were treatment related. Objective responses were observed in patients treated with a dose of 1 mg/kg or higher. Responses were noted in patients with melanoma, NSCLC, RCC, or ovarian cancer but none were observed in CRC or pancreatic cancer. Treatment was well tolerated with the expected immune-related adverse events, which were mostly grade 1 or 2 and were managed by interruption of treatment or with glucocorticoids. Despite encouraging results, BMS-936559 does not seem to have been developed any further by the manufacturer.

MEDI4736 is a humanized PD-L1-blocking mAb [45] that showed T cell-dependent antitumor activity in an *in vivo* model where tumor cells were coimplanted with human T cells or when a surrogate antibody was used in a syngeneic mouse model (Table 4). Combination with oxaliplatin resulted in complete tumor regression in greater than 50% of treated animals. MEDI4736 is being studied in a Phase I trial in advanced solid tumors with expansion cohorts in several responding tumor types including NSCLC and melanoma [46,47]. It was noted to be well tolerated, with tumor shrinkage detectable in many tumor types. It is also being studied in combination with tremelimumab (a mAb to CTLA-4) in patients with advanced solid tumors [48] and with dabrafenib and trametinib or trametinib alone in patients with malignant melanoma.

Other mAbs to PD-L1 include MPDL3280A, which has shown impressive results shrinking tumors in 43% of patients in a Phase I clinical trial in metastatic urothelial bladder cancer [49] (http://www.roche.com/media/media_releases/med-cor-2014-05-31.htm) (Table 4). It has been granted FDA breakthrough designation due to its activity in urothelial bladder cancers. It has also shown acceptable tolerability and efficacy in several solid tumors, with no MTD reached [50]. Ongoing clinical trials include Phase I trials in advanced solid tumors and the phase II BIRCH trial and phase III OAK trial in NSCLC. MSB0010718C is another mAb to PD-L1. It is a fully human IgG1 and is expected to show antineoplastic activity by inhibiting the PD-1/PD-L1 interaction and by antibody-dependent cell-mediated cytotoxicity [51]. MSB0010718C is being studied in a Phase I trial in refractory malignancies.

Clinical trials of PD-L2 Ig fusion protein

As an alternative strategy to mAbs, AMP-224 (B7-DC-Ig) was developed as a chimeric fusion protein between the extracellular domain of PD-L2 and an Fc portion of IgG2a (http://www.prnewswire.com/news-releases/glaxosmithkline-and-amplimmune-form-global-strategic-collaboration-99938599.html). *In vivo* studies suggested that this fusion protein can ameliorate disease by inducing immune responses to pathogens. Furthermore, the murine form of AMP-224 can enhance the therapeutic efficacy of vaccination when combined with cyclophosphamide in a mouse model [52]. AMP-224 exerts its therapeutic effect via a mechanism distinct from the direct blocking of the PD-1/PD-L1 interaction. It is hypothesized that AMP-224 can deplete exhausted effector T cells that express high levels of PD-1 and the T cell pool is replenished with functional T cells [53]. Ongoing trials with AMP-224 include a Phase I trial with cyclophosphamide in advanced solid tumors that has shown no drug-related inflammatory adverse events other than infusion reactions, as well as preliminary tumor responses [53].

Concluding remarks

After success with ipilimumab in the treatment of malignant melanoma, the field of cancer immunotherapy continues to grow. Blockade of T cell inhibition allows restored antitumor immunity and has shown impressive results in clinical trials. Beyond the CTLA-4 pathway, T cell inhibition mediated by the PD-1/PD-L1 pathway is now the most studied and clinically developed cancer immunotherapy. Several mAbs to PD-1 or PD-L1 have been studied in Phase I trials and continue to be evaluated in Phase II and Phase III trials. These therapies have been well tolerated, with no MTD reached in most single-agent Phase I studies. Grade 3 or 4 adverse event rates have been generally acceptable, with low treatment-related discontinuation rates. Response rates have been impressive, particularly in melanoma, NSCLC, and RCC, with encouraging early reports from urothelial bladder cancer and platinum-resistant

Box 1. Outstanding questions

- Why are the response rates of anti-PD-1 and anti-PD-L1 variable among different cancers?
- Can clinical response biomarkers be identified and how can these be integrated into clinical practice?
- How can anti-PD-1 and anti-PD-L1 antibodies be integrated into current treatment regimens in upfront and relapsed settings?
- Does PD-1 expressed on immune cells other than T cells play a role in anti-PD-1/PD-L1 therapy?
- Can we develop small-molecule inhibitors of the PD-1/PD-L1 interaction?

ovarian cancer. PD-1/PD-L1 blockade has also shown efficacy in hematologic malignancies. What has been striking is the durability of responses. Potential biomarkers for efficacy of PD-1/PD-L1 blockade are being studied, to date mainly focusing on PD-L1 expression by the tumor. The data from most clinical studies are not yet mature, but preliminary data indicate that PD-L1 expression by tumor cells may correlate with higher response rate and PFS in some patients. It is encouraging that pembrolizumab has been granted accelerated FDA approval as second-line therapy in advanced melanoma. Integrating immunotherapy into current clinical practice remains to be studied and many outstanding questions remain (Box 1). The variability in response both by tumor subtype and by PD-L1 expression indicates that immune checkpoint inhibition remains a field that is open to study and that PD-1/PD-L1 blockade, like CTLA4 blockade before it, is only the beginning of immunomodulatory therapies.

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Biology of Human Tumors

Clinical Cancer Research

Expression, Clinical Significance, and Receptor Identification of the Newest B7 Family Member HHLA2 Protein

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Abstract

Purpose: HHLA2 (B7H7/B7-H5/B7y) is a newly identified B7 family member that regulates human T-cell functions. However, its protein expression in human organs and significance in human diseases are unknown. The objective of this study was to analyze HHLA2 protein expression in normal human tissues and cancers, as well as its prognostic significance, to explore mechanisms regulating HHLA2 expression, and to identify candidate HHLA2 receptors.

Experimental Design: An immunohistochemistry protocol and a flow cytometry assay with newly generated monoclonal antibodies were developed to examine HHLA2 protein. HHLA2 gene copy-number variation was analyzed from cancer genomic data. The combination of bioinformatics analysis and immunologic approaches was established to explore HHLA2 receptors.

Results: HHLA2 protein was detected in trophoblastic cells of the placenta and the epithelium of gut, kidney, gallbladder, and breast, but not in most other organs. In contrast, HHLA2 protein was widely expressed in human cancers from the breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney, and esophagus. In a cohort of 50 patients with stage I–III triple-negative breast cancer, 56% of patients had aberrant expression of HHLA2 on their tumors, and high HHLA2 expression was significantly associated with regional lymph node metastasis and stage. The Cancer Genome Atlas revealed that HHLA2 copy-number gains were present in 29% of basal breast cancers, providing a potential mechanism for increased HHLA2 protein expression in breast cancer. Finally, Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) was identified as one of the receptors for HHLA2.

Conclusions: Wide expression of HHLA2 in human malignancies, together with its association with poor prognostic factors and its T-cell coinhibitory capability, suggests that the HHLA2 pathway represents a novel immunosuppressive mechanism within the tumor microenvironment and an attractive target for human cancer therapy. *Clin Cancer Res; 21(10); 2359–66. ©2014 AACR.*

See related commentary by Xiao and Freeman, p. 2201

Introduction

The past decade has witnessed an important change in the understanding of T-cell biology and tumor immunology with the recognition of immune checkpoints through the B7–CD28 pathways (1–3). B7-1/B7-2/CD28/CTLA-4 is the prototypic B7–CD28 pathway. This loop of costimulation and coinhibition is critical for regulating the early stages of T-cell responses in lymphoid organs. Several additional B7 family members are believed to play important roles in peripheral tolerance and tumor immune evasion—PD-L1 [B7-H1 (4, 5)], B7-H3 (6), and B7x [B7-H4/B7S1 (7–9)]. The ligands PD-L1, B7-H3, and B7x function by inhibiting effector T cells in peripheral tissues (10, 11). These ligands are expressed in various human cancers and their expression can lead to immune tolerance in the tumor microenvironment by inhibiting T-cell proliferation and function (1, 12–14). In addition, B7x can interact with myeloid-derived suppressor cells (15, 16), which may also promote tumor growth. Clinically, higher expression of these ligands is associated with a poor prognosis in various malignancies. On the basis of these functional and clinical observations, blocking some of the B7–CD28 pathways has yielded some therapeutic success in human malignancies. The anti-CTLA-4 antibody achieved clinical responses in melanoma (17), whereas anti-PD-L1 or anti-PD-1 antibodies have shown responses in melanoma, renal cell cancer, and non–small cell lung cancer (18–20). The therapeutic responses seen in these patients are durable and they are longer than chemotherapy or other targeted agents.

HERV–H LTR-associating 2 (HHLA2, also called B7H7/B7-H5/B7y) has been recently discovered as the newest member of the B7

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The B7 and CD28 families are critical in regulating T-cell responses, and antibodies against PD-1, PD-L1, and CTLA-4 have successfully led to durable clinical remissions in various malignancies. HHLA2 was recently discovered as a new member of the B7 family and shown to regulate T-cell function. Here, we showed that HHLA2 had limited expression in normal human tissues but was expressed in various human cancers. We demonstrated that its expression on tumor cells was associated with increased lymph node metastases in limited-stage triple-negative breast cancer (TNBC). Analyzing the TCGA database revealed that TNBC had higher HHLA2 copy-number gains than other subtypes of breast cancer, providing a mechanism of overexpression. Finally, we identified one of the receptors for HHLA2. This is the first study to report HHLA2 expression in human cancers and clinical significance and, together with its previously reported T-cell coinhibition properties, shows that this could contribute to tumor immune suppression and be a target for cancer immunotherapy.

family (21–23) and has 23% to 33% similarity in amino acid sequence with the other B7 molecules (21). This ligand is the only B7 family member that is found in humans but not in mice. It is constitutively expressed on the surface of human monocytes and is induced on B cells. HHLA2 binds to its putative receptor(s) on a variety of immune cells, including CD4 and CD8 T cells and antigen-presenting cells (21). Similarly to B7-H3, both a T-cell coinhibitory role and a costimulatory role have been reported for this ligand (21, 22). There have been no reports published on the protein expression of HHLA2 in normal human tissues or cancers, and the clinical significance of this ligand is not currently understood. Here, we present the first study on the expression of this protein in peripheral tissues as well as on numerous human cancers. Furthermore, we have demonstrated that the overexpression of this protein in tumors is associated with worse clinical outcomes in patients with breast cancer. One of the mechanisms whereby HHLA2 is overexpressed in human cancer seems to be gene copy-number variation (CNV). Finally, we discovered that Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) is one of the receptors for HHLA2.

Patients and Methods

Patients and samples

Normal and cancerous tissue microarrays were purchased from Imgenex Corp for the analysis of HHLA2 expression in human tissues. For the TNBC cohort, 50 cases were selected from our tumor registry between 2002 and 2011 who were diagnosed with local or locally advanced breast cancer diagnosed at our institution between 2002 and 2011. All of these patients underwent surgery as the primary treatment followed by chemotherapy or radiotherapy or both. Tissue blocks from the mastectomy or lumpectomy specimen were located and slides were made from the same section. This was done so as to enable us to determine whether HHLA2 expression in the primary tissue is associated with prognostic features. Using retrospective chart review, the relevant clinical data were collected from the files. Hormone receptor status and Her2 status were also obtained by retrospective review. All protocols were reviewed and approved by the Institutional Review Board.

Monoclonal antibodies and immunohistochemistry

A mouse monoclonal antibody against HHLA2 (Clone 566.1, IgG1) and 3T3 cell lines expressing HHLA2 or CTLA-4 were recently generated (21). Cell blocks of the 3T3 cells expressing either HHLA2 or CTLA-4 were prepared for IHC controls by fixing the cells with 4% paraformaldehyde and then embedding them in HistoGel (Thermo Scientific). These samples were then embedded in paraffin and cut onto slides and used as positive and negative controls, respectively. These controls were then stained for HHLA2 using our mAb. Briefly, 4 to 5-μm thick formalin-fixed paraffin-embedded specimen slides were used. The tissue sections were deparaffinized in xylene and dehydrated through graded alcohols to water. The samples were boiled in citrate buffer (pH, 6.0) using a microwave for 2 minutes and then incubated in an antigen retrieval steam chamber between 80°C and 100°C for 30 minutes. To block endogenous peroxidase activity, all of the sections were treated with 3% hydrogen peroxide for 10 minutes. Nonspecific binding of IgG was blocked by using serum-free protein block solution (Dako). The sections were incubated with anti-HHLA2 mAbs for 30 minutes. They were then incubated with the Dako envision+ HRP-labeled anti-mouse polymer. Signals were generated by incubation with 3,3′-diaminobenzidine. Finally, the sections were counterstained with hematoxylin and observed under the microscope. The slides were reviewed in tandem by a breast pathologist and by an oncology physician trained in breast pathology.

Cell lines and FACS

Cell lines were cultured in either complete RPMI or DMEM. Cells were stained with anti-HHLA2 mAb 566.1 and then with a secondary anti-mouse IgG-APC–conjugated antibody. For receptor binding, 3T3 cells expressing HHLA2-YFP or CTLA-4-YFP were incubated with TMIGD2-Ig or control Ig for 45 minutes on ice and then stained with anti-human IgG-APC. Similarly, 3T3 cells expressing TMIGD2-YFP or CTLA-4-YFP were incubated with HHLA2-Ig or control Ig for 45 minutes on ice and then stained with anti-human IgG-APC. Samples were analyzed by a BD FACSCalibur flow cytometer and FlowJo software.

Bioinformatic analysis

The NCBI database was queried for proteins of the immunoglobulin family with homologs in humans and monkeys, but not in mice or rats. The sequences of the resulting list of proteins were analyzed by various domain-prediction programs to determine if these proteins contained Ig, IgC, IgC-like, IgV, or IgV-like domains. The list was further refined by excluding proteins that did not contain a transmembrane domain. MacVector 10.6. was used for sequence alignment and homology comparison. The phylogenetic tree was generated by PAUP (4.0b10) after removal of significant inserts and trimming C- and N-terminal extensions from sequence alignments (24). Motifs and domains were analyzed with EMBL-EBI tools, SMART, and CBS Prediction. For gene CNVs, the cBioPortal for Cancer Genomics database and TCGA were analyzed.

Fusion protein production and purification

TMIGD2-Ig fusion protein was prepared by PCR-amplifying the extracellular domain of the protein without the signal peptide.

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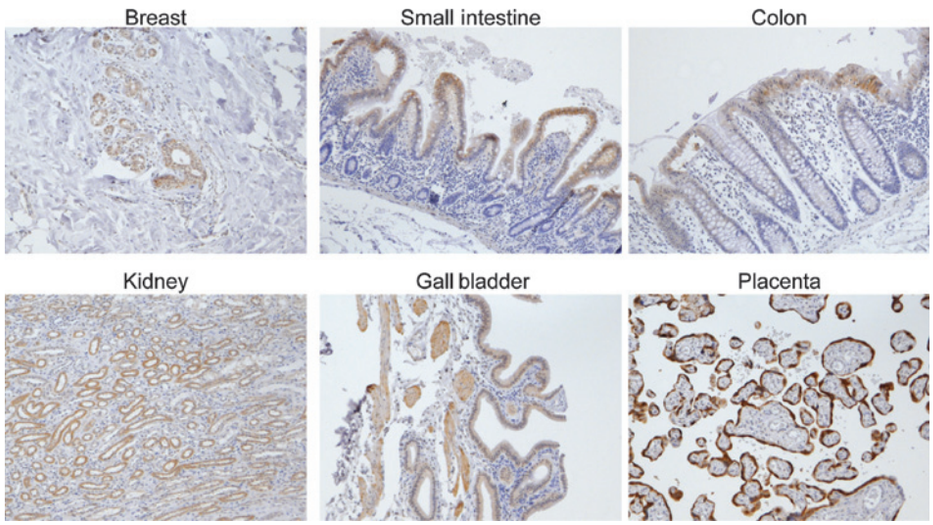

Figure 1.
Normal human tissues that stained positively for expression of HHLA2 protein. Images were acquired at ×10. A summary of staining results from tissue microarrays is shown in Table 1.

The amplified product was inserted into a human IgG1 Fc tag of plasmid pMT/BiP as described previously (7). *Drosophila* Schneider 2 cells were cotransfected with this construct and a plasmid inducing hygromycin resistance. The fusion protein was expressed in Express Five serum-free medium (Life Technologies) and purified using Protein G Plus Agarose columns (Pierce). The purity of the fusion protein was confirmed by SDS-PAGE.

Statistical analysis

For continuous variables, the *t* test was used when the normality assumption was met and the rank-sum test and median was calculated for those, which violated normality. The χ^2test was used to analyze the association between categorical variables and if the expected frequency was less than 5 in more than 20% of the cells, the Fisher exact test was used. All *P* values < 0.05 were considered statistically significant.

Results

Normal human tissue expression of HHLA2

The expression of HHLA2 protein in human tissues is unknown at present. To examine the protein expression, we used our recently generated HHLA2-specific monoclonal antibody clone 566.1 to develop an immunohistochemistry protocol to detect HHLA2 in formalin-fixed paraffin-embedded specimens in which 3T3 cells expressing HHLA2 or CTLA-4 served as positive and negative controls, respectively (Supplementary Fig. S1). We used this technique to stain tissue microarrays of normal human organs (Table 1). Our results demonstrated that the majority of normal organs did not express HHLA2 at the protein level; however, trophoblastic cells of the placenta and epithelial cells of the gut, kidney, gallbladder, and breast expressed this ligand (Fig. 1 and Table 1). Although primary and secondary lymphoid organs were largely negative, a few scattered cells appeared to stain positively in these samples. These results reveal that endogenous HHLA2 protein is absent in most normal tissues, but mainly expressed on epithelial cells of a few tissues.

HHLA2 is widely expressed in various human cancers

HHLA2 is able to inhibit both human CD4 and CD8 T-cell functions (21), therefore, it is possible that human cancer may exploit this pathway as an immune-evasion mechanism. As no knowledge currently exists about HHLA2 protein in human cancer tissues, we next examined the expression of HHLA2 in human cancer tissues using IHC staining of tissue microarrays of common cancers from various organs (Fig. 2). HHLA2 was expressed in 50% or more of cancer samples from the breast (7 of 10), lung (6 of 9), thyroid (6 of 9), melanoma (5 of 9), ovary (4 of 8), and pancreas (5 of 10) (Table 2). The localization of the protein was membranous and cytoplasmic in tumor cells (Fig. 2). HHLA2 protein was also expressed in cancers of the liver, bladder, colon, prostate, kidney, and esophagus (Table 2 and Fig. 2). In addition, we found that of the 20 human cell lines examined in breast cancer and hematologic malignancies of leukemia and lymphoma, 12 expressed HHLA2 on their surface by flow cytometry (Supplementary Table S1). These results demonstrate that HHLA2 protein is widely overexpressed in human cancers and has a high prevalence in certain malignancies.

Table 1. HHLA2 protein expression in normal human organs assessed by immunohistochemistry on tissue microarrays

Normal tissues (number positive/total cores analyzed)		
Placenta (3/3) (trophoblastic cells)	Colon (3/3) (epithelial cells)	Breast (3/3) (ductal epithelium)
Small intestine (2/2) (epithelial cells)	Kidney (4/5) (tubular epithelium)	Gallbladder (5/11) (epithelial cells)
Adrenal gland (0/2)	Aorta (0/2)	Brain (0/7)
Esophagus (0/2)	Larynx (0/9)	Liver (0/3)
Lung (0/3)	Lymph node (0/12)	Ovary (0/1)
Pancreas (0/2)	Prostate (0/3)	Skin (0/3)
Spleen (0/3)	Stomach (0/3)	Subcutis (0/2)
Thymus (0/2)	Thyroid (0/2)	Tonsils (0/2)
Umbilical cord (0/2)	Uterine cervix (0/4)	Uterus (0/9)

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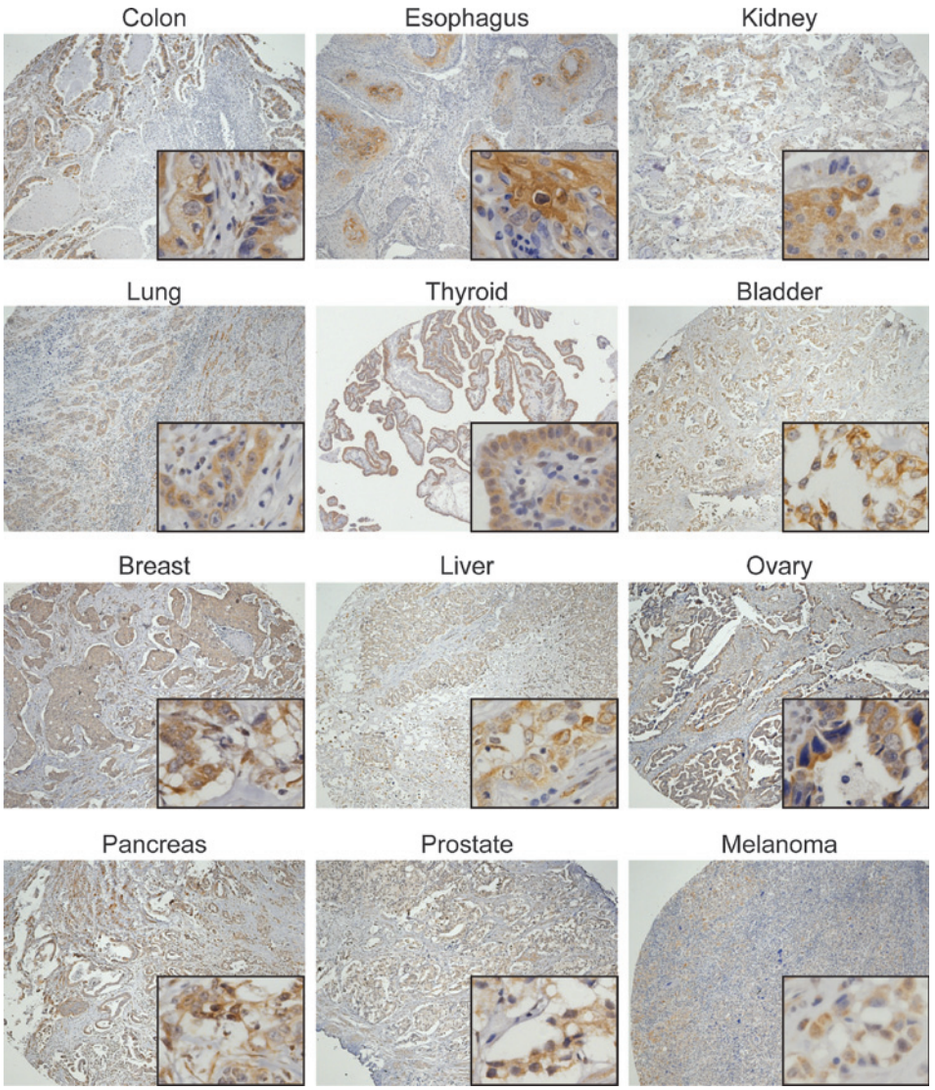

Figure 2.
Examples of human cancers that stained positively for HHLA2 protein expression. Full-size images were acquired at ×5, and zoom-in images were originally acquired at ×40. A summary of the tissue microarray staining data is shown in Table 2.

HHLA2 expression in triple-negative breast cancer

We also evaluated HHLA2 expression in a cohort of 50 patients with early-stage TNBC because of the limited therapeutic options for this breast cancer subtype. The characteristics of the patient population are shown in Table 3. The mean age of the patient population was 57.6 years, average tumor size was 2.35 cm (SD, 1.49), 70% had at least one positive lymph node ($n = 35$), and the distribution of American Joint Committee on Cancer (AJCC) Stage was stage I in 20%, stage II in 50%, and stage III in 30%. All patients underwent primary therapy (i.e., mastectomy or lumpectomy and radiotherapy), and most of them (92%) received adjuvant chemotherapy.

Table 2. HHLA2 protein expression in human cancers assessed by immunohistochemistry on tissue microarrays

Cancer samples (number positive/total cores)		
Breast (7/10)	Lung (6/9)	Thyroid (6/9)
Malignant melanoma (5/9)	Pancreas (5/10)	Ovary (4/8)
Liver (4/10)	Bladder (4/10)	Colon/rectum (3/8)
Prostate (3/9)	Kidney (2/6)	Esophagus (2/10)
Endometrial (0/9)	Gallbladder (0/10)	Larynx (0/10)
B-cell lymphoma (0/10)	Stomach (0/10)	Uterine cervix (0/10)

Using IHC staining, we found that within a given TNBC tumor, the expression and intensity of HHLA2 protein was quite homogeneous throughout the tumor. Hence, HHLA2 expression was graded on the basis of intensity of staining between 0 and 3. Grades of 0 and 1 had no or minimal staining, respectively, whereas 2 and 3 had moderate and strong membranous/cytoplasmic staining. Zero and 1 were considered to be negative or low expression, whereas 2 and 3 were considered to be high expression (Fig. 3).

In the 50 TNBC samples stained, HHLA2 was graded as 0 in 12% ($n = 6$), 1 in 32% ($n = 16$), 2 in 40% ($n = 20$), and 3 in 16% ($n = 8$) of the tissue sections (Table 3). When classified as a binary variable, 56% ($n = 28$) exhibited high (grades 2 or 3) and 44% ($n = 22$) had low expression (grades 0 or 1) of HHLA2. In the bivariate analysis, high HHLA2 expression was associated with lymph node positivity (0 vs. ≥ 1, $P = 0.04$) and advanced stage of the disease ($P = 0.03$) at the time of diagnosis, features known to be associated with an increased risk of recurrence. HHLA2

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Table 3. Clinicopathologic features of HHLA2 expression in a human TNBC cohort

Demographics	Entire population	Population by HHLA2 expression		*P*
		High ($n = 28$)	Low ($n = 22$)	
Mean age (95% CI)	57.6	57.1 (52.1–62.1)	58.4 (53–63.8)	0.71
Mean size (25th–75th percentile)	2.4	2.6 (1.8–4)	2.15 (1.4–2.5)	0.12
Lymph nodes, *n* (%)				0.04
0	15 (30%)	5 (18%)	10 (46%)	
≥1	35 (70%)	23 (82%)	12 (54%)	
AJCC stage				0.03
I	10 (20%)	2 (7%)	8 (36%)	
II	25 (50%)	15 (54%)	10 (46%)	
III	15 (30%)	11 (39%)	4 (18%)	

expression was not related to age or to the size of the tumor. Together, these data demonstrate that more than half of TNBC tumors have HHLA2 overexpression and that patients with higher levels of HHLA2 on their tumors are significantly more likely to have the disease spread and at an advanced stage.

HHLA2 gene copy number variations in triple-negative breast cancer

HHLA2 was overexpressed at the protein level in breast cancer, but the mechanism(s) upregulating the expression in cancer cells is unknown. Therefore, we sought to determine whether gene amplification was a potential mechanism of overexpression. By analyzing the cBioPortal for Cancer Genomics database (25, 26), we found that HHLA2 gene alterations were present in 18.8% and 23% of all cases with breast cancer in TCGA (27) and in the TCGA provisional database studies, respectively. Because TNBC is predominantly comprised of the basal subtype, we compared the copy-number gain of the HHLA2 locus in basal with nonbasal breast cancer using the TCGA registry. HHLA2 was altered in 32% of the basal subtype, which is almost twice the frequency observed in all breast cancers independent of their subtype (18%). The vast majority of HHLA2 CNVs in basal breast cancers were amplifications or gains (>95%). Hence, given that HHLA2 protein upregulation is present in approximately 56% of our samples, our results suggest that one mechanism of up-regulation of HHLA2 protein in TNBCs is gene copy-number gain.

Identification of transmembrane and immunoglobulin domain–containing protein 2 as one of the receptors for HHLA2

Receptors for HHLA2 are widely expressed on both naïve and activated T cells as well as dendritic cells, monocytes, and B cells (21). As HHLA2 is a member of the immunoglobulin superfamily and has orthologs in humans and monkeys but not in mice or rats, we hypothesized the receptors for HHLA2 may belong to the immunoglobulin superfamily and have the same phylogenetic pattern due to coevolution.

From more than 500 members of the immunoglobulin superfamily (28, 29), a list was compiled of the immunoglobulin family members expressed in humans and monkeys but not in mice or rats. This list was further refined by only including members with predicted transmembrane domains and we then stably transfected these candidates into 3T3 cells. We tested their ability to bind to the HHLA2-Ig fusion protein. Using flow cytometry, we found that HHLA2-Ig bound to cells expressing Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2, Fig. 4A and B). A TMIGD2-Ig fusion protein was then constructed in which the extracellular domain of TMIGD2 was linked to the Fc region of human IgG1 (TMIGD2-Ig). TMIGD2-Ig bound strongly to 3T3 cells expressing HHLA2 but not CTLA-4 by flow cytometry (Fig. 4B). TMIGD2 contains an N-terminal signal peptide, an extracellular IgV-like domain, three potential sites for N-linked glycosylation, a transmembrane region, and a cytoplasmic tail with four potential sites for phosphorylation, and a possible site for SH3 domain recognition (Fig. 4A). By sequence analysis, we found TMIGD2, the immunoglobulin-containing and proline-rich receptor-1 (IGPR-1; ref. 30), and CD28 homolog (CD28H; ref. 22) are the same molecule. IGPR-1 was originally identified as a adhesion molecule involved in angiogenesis (30), whereas CD28H was recently reported as a receptor by a high-throughput screen of transmembrane proteins (22). Thus, TMIGD2/IGPR-1/CD28H is one of the receptors for HHLA2.

Discussion

The B7 ligand family and the CD28 receptor family are the major driving force of T-cell costimulation and coinhibition. These molecules have been intensely studied for their potential clinical impact in human malignancies, especially with regard to ectopic tumor cell expression of negative coinhibitory molecules. Here, we present the first study on the protein expression, clinical significance, and mechanism of upregulation of HHLA2 in human tissues and cancers. The results reveal that HHLA2 is a suitable target for cancer therapy.

HHLA2 appears to have limited expression in normal tissues. Most human tissues we tested were negative for HHLA2 protein. A

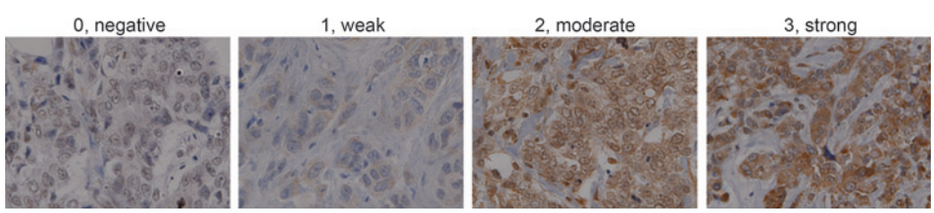

Figure 3.
HHLA2 expression in human TNBC. Tumors from a cohort of patients with TNBC were stained for HHLA2 protein expression. The level of HHLA2 protein was graded as follows: 0, absent staining; 1, weak to minimal staining; 2, moderate staining; 3, strong staining.

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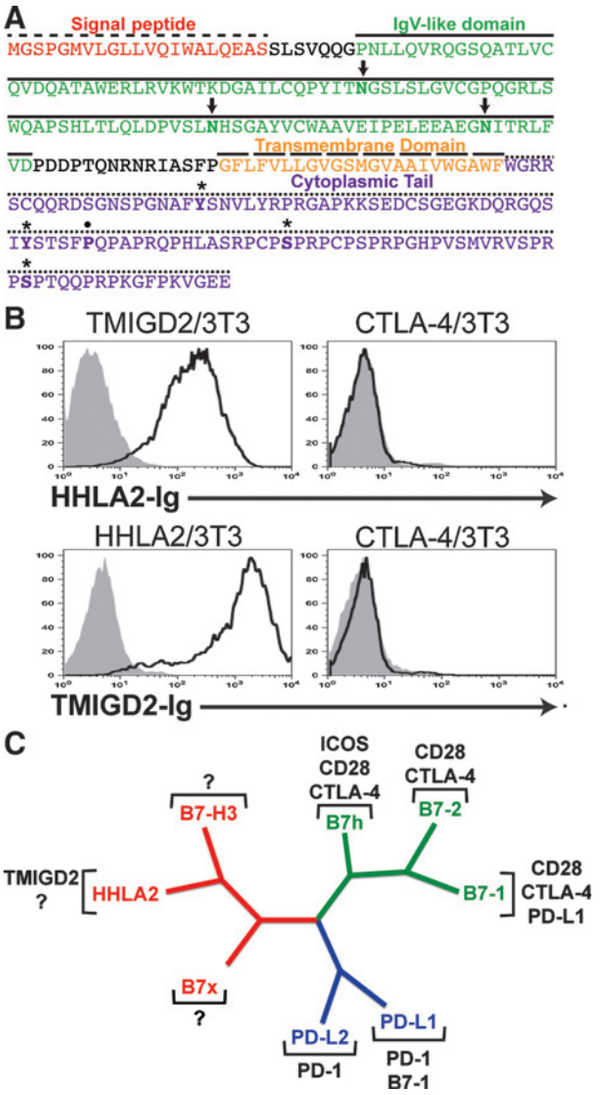

Figure 4.
TMIGD2 is an immunoglobulin family member that binds to HHLA2. A, TMIGD2 is composed of a signal peptide, a single extracellular immunoglobulin domain, a transmembrane domain, and a cytoplasmic tail. There are three predicted sites of N-linked glycosylation within the Ig domain (arrows) and four sites of predicted phosphorylation (asterisks) and one potential SH3-binding domain (dot) within the cytoplasmic tail. B, TMIGD2-Ig binds to HHLA2-expressing 3T3 cells but not to CTLA-4–expressing 3T3 cells. Cells were stained with HHLA2-Ig (open histograms) or control Ig (shaded histograms) followed by anti-human IgG-APC. C, phylogenetic tree of the human B7 and CD28 families. The phylogenetic comparison of human B7 molecules was generated by PAUP and was divided into three groups. Receptors for human B7 molecules are also shown.

few organs, including intestines, breast, kidney, gallbladder, and placenta, express HHLA2, particularly on epithelial cells. We previously showed that blood B cells activated by LPS and IFN expressed HHLA2 (21). IHC staining of tissue microarrays of normal human organs showed there were only scattered HHLA2-positive cells in primary and secondary lymphoid organs. Currently, it is unknown whether activated B cells in blood and in tissues have different expression patterns of HHLA2. Tissue-expressed PD-L1, another B7 family member, was recently shown to protect against gut inflammation in mouse models (31). Future investigation is warranted to determine whether HHLA2 in the intestines contributes to the immune tolerance or intestinal inflammation. We observed high expression of HHLA2 in the placental tissue, suggesting that it may play a role in fetal maternal immune tolerance. Interestingly, HHLA2 polymorphisms are associated with Autism spectrum disorders, a disease whose etiology remains poorly understood (32). Immune dysregulation was recently proposed to contribute to the rapid development of Autism spectrum disorder in genetically susceptible children (32). Further study will also be required to determine whether HHLA2 is involved in the development of Autism spectrum disorder.

HHLA2 was highly expressed in most malignant tissues. This wide expression in various tumors indicates that HHLA2 expression may be a critical step in tumor evolution and that HHLA2 could confer a survival advantage to tumors via suppression of host antitumor immunity. Because breast cancer had a high expression of HHLA2, we evaluated its clinical impact in our TNBC cohort. Analysis of HHLA2 expression in the breast cancer cohort revealed that HHLA2 was highly expressed in 56% of TNBC patients; about 80% of TNBCs are basal subtype by gene expression. Tumors with high HHLA2 expression exhibited uniform expression of HHLA2 in the membrane or cytoplasm with minimal intratumoral heterogeneity. In contrast, other B7 ligands such as PD-L1 show focal expression and significant tumor heterogeneity. This uniform expression of HHLA2 in tumors suggests that it may be a primary change in tumors during tumor evolution and/or its expression could be induced by factors in the tumor microenvironment. There are at least two possible mechanisms governing the upregulation of HHLA2 expression: inflammatory stimulation and gene copy-number gain. HHLA2 expression is induced on B cells and enhanced on monocytes by stimulation with LPS and IFNγ (21). Our gene copy-number analysis revealed that the vast majority of HHLA2 CNVs in basal breast cancer were gains (>95%), suggests that gene copy-number gain is one of the mechanisms upregulating HHLA2 expression in cancer. Expression of HHLA2 was associated with two prognostic factors—advanced stage and lymph node positivity—but it was not related to the size of the tumor by bivariate analysis. The association of HHLA2 with lymph node–positive disease suggests that this may be a change that is required for early tumor invasion.

We have previously reported that receptors for HHLA2 exist on a wide variety of immune cells, including T cells, B cells, monocytes, and DCs. We utilized bioinformatics analysis and immunology approaches to determine that TMIGD2/IGPR-1/CD28H is one of the receptors for HHLA2. This strategy was used because of the unique phylogenetic profile of HHLA2 and the observation that ligand and receptor pairs tend to coevolve. It is unlikely, however, that tumor-expressed HHLA2 inhibits the immune system through the interaction with TMIGD2/IGPR-1/CD28H, as CD28H is reported to be expressed on naïve T cells but not on other immune cells and is lost rapidly after activation of naïve T cells (22), whereas tumor-infiltrating T cells are not naïve cells. Further study will be required to discover coinhibitory receptors on immune cells that tumor cell-expressed HHLA2 interacts with to inhibit anticancer immunity.

Because this is a cross-sectional study and this is a small cohort of TNBC patients analyzed and hence, limited inference could be

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drawn from this cross sectional study. The association of HHLA2 positivity and advanced stage could have been driven by the association of HHLA2 with lymph node positivity. Hence, larger studies are needed to confirm these associations and other important characteristics like disease-free and overall survival in triple negative and other breast cancers.

In summary, HHLA2 has restricted tissue expression, but is overexpressed in various human cancers. Importantly, its expression is associated with important prognostic factors in breast cancer. Furthermore, cancer cells may amplify the HHLA2 gene to increase its expression and to undermine host immunity. Finally, our results suggest that HHLA2 in human cancers is a suitable target for cancer immunotherapies such as checkpoint blockade and antibody–drug conjugate treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Janakiram, A. Zhao, J.A. Sparano, X. Zang
Development of methodology: M. Janakiram, J.M. Chinai, R. Zhao, A. Zhao, S.C. Almo,
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Janakiram, J.M. Chinai, S. Fineberg, J.A. Sparano
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Janakiram, J.M. Chinai, S. Fineberg, A. Fiser, C. Montagna, K.C. Ohaegbulam, A. Zhao, J.A. Sparano
Writing, review, and/or revision of the manuscript: M. Janakiram, J.M. Chinai, S. Fineberg, E. Castano, A. Zhao, J.A. Sparano, X. Zang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Janakiram, R. Medavarapu, H. Jeon, R. Zhao,
Study supervision: S. Fineberg, J.A. Sparano, X. Zang
Other (reviewed cases, gathered information about patients): R. Medavarapu
Other (reviewed cases as a pathologist): E. Castano
Other (developed the antibody used in the article): R. Zhao

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HHLA2 and TMIGD2: new immunotherapeutic targets of the B7 and CD28 families

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Keywords: angiogenesis, B7 family, CD28 family, HHLA2, immunosuppression, immunotherapy, ligand, receptor, tumor microenvironment, TMIGD2

We and others recently discovered HHLA2 as a new B7 family member and transmembrane and immunoglobulin domain containing 2 (TMIGD2) as one of its receptors. Based on a new study we propose that HHLA2 may represent a novel immunosuppressive mechanism within the tumor microenvironment and hence could be a target for cancer therapy. TMIGD2 may be another therapeutic target.

HHLA2 (B7H7/B7-H5/B7y) has recently been identified as a new member of the B7 family member.[1-3] HHLA2 was initially discovered as a gene in the Immunoglobulin (Ig) superfamily when screening the human genome for human endogenous retroviral (HERV) long terminal repeat (LTR) sequences which provide polyadenation signals.[4] Hence the name, HHLA2, is short for HERV-H LTR-associating 2. HHLA2 orthologs appear to be present in a wide range of species such as fish, frog, giant panda, monkey and human, but not in laboratory mouse and rat strains. The HHLA2 protein has amino acid similarity of 23 to 33% to the other human B7 family molecules and phylogenetically it is most similar to B7-H3 and B7x (B7-H4/B7S1). The predicted structure of HHLA2 is a type I transmembrane molecule with three extracellular Ig domains. This is unique as most other B7 family members contain only two Ig domains while human B7-H3 has four Ig domains (**Fig. 1A**).

HHLA2 functions as a T cell coinhibitory molecule as it suppresses proliferation and cytokine production of both human $CD4^+$ and $CD8^+$ T cells.[1,5] HHLA2 is constitutively expressed on the surface of human monocytes and is induced on B cells after stimulation.[1] Unlike PD-L1 and B7–1 though, HHLA2 is not inducible on T cells. The differences in expression on immune cells suggest that HHLA2 could be involved in immune regulation at a different functional level than other B7 family members. Using immunohistochemistry with an HHLA2 monoclonal antibody, we have recently found that HHLA2 is not expressed in most human tissues, except the placenta, kidney, intestine, gall bladder, and breast.[6] Expression of HHLA2 in the placenta and the intestines is interesting as it may help fetal-maternal immune tolerance or control intestinal inflammation, respectively. Importantly, we have shown many human cancers overexpress HHLA2 including cancers from the breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney, and esophagus.[6] Moreover, in a small cohort of human triple-negative breast cancer (TNBC) patients, higher expression of HHLA2 on tumor cells was associated with increased lymph node metastases.[6] The wide expression of HHLA2 in human cancers and its association with more invasive disease in the TNBC cohort suggest that HHLA2 potentially plays an important role in tumor evolution and metastases through immune suppression.

There are at least two mechanisms upregulating HHLA2 expression. One mechanism is inflammatory stimulation.[1,2] HHLA2 expression can be increased on monocytes and macrophages and is induced on B cells by stimulation with LPS and IFN-γ.[1,2] The second mechanism is the gene copy-number variation.[6] We compared gene dosage in the basal subtype of TNBC using the cBioPortal for the Cancer Genomics database.[7] In this subtype, 32% had HHLA2 gene copy-number variations and the majority (95%) of these variants were either amplifications or gains of HHLA2 gene copy number,[6] suggesting this could be another mechanism of overexpression.

Receptors for HHLA2 can be found on a wide variety of immune cells, including T cells, B cells, monocytes, and dendritic cells.[1] We and others have independently identified one of receptors for HHLA2, TMIGD2,[6] also called CD28 homolog (CD28H),[2] through a bioinformatics analysis/immunological approach and a high-throughput screening, respectively. Like HHLA2, this molecule is expressed in humans and monkeys but not in mice or rats. This molecule was initially reported as an endothelial adhesion molecule which was renamed Immunoglobulin-containing and Proline-rich

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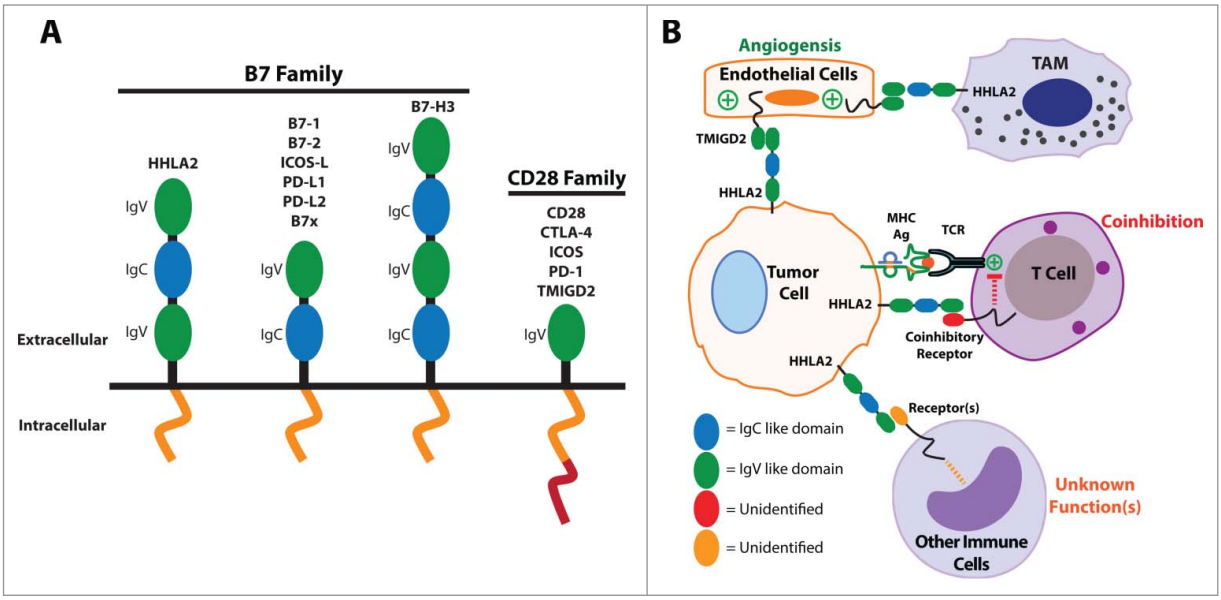

Figure 1. The B7 and CD28 families and the significance of HHLA2 and TMIGD2 within the tumor microenvironment. (**A**) A structural representation of the B7 and CD28 family members. (**B**) A proposed model for the roles of HHLA2 and TMIGD2 within the tumor microenvironment. Tumor-expressed HHLA2 can interact not only with an unidentified receptor on activated T cells that leads to coinhibition, but also with TMIGD2 on endothelium that stimulates tumor angiogenesis. Additionally, tumor-expressed HHLA2 can bind to other immune cells and likely affects their functions in ways that are not yet understood. Finally, tumor-associated macrophages (TAM) may express HHLA2 and interact with TMIGD2 on endothelium.

Receptor-1 (IGPR-1).[8] TMIGD2 protein can be detected in cells of epithelial and endothelial origins, and is able to enhance angiogenesis *in vitro* when overexpressed by endothelial cell lines.[8] Furthermore, TMIGD2 is reported as a stimulatory receptor expressed primarily on naive T cells.[2] Like other CD28 family members, TMIGD2 is an Ig superfamily member with an extracellular IgV-like domain, a transmembrane region, and a cytoplasmic tail.[6] The cytoplasmic tail contains tyrosine residues which can be phosphorylated[2] and a proline-rich domain which associates with multiple Src homology 3 (SH3)-containing signaling molecules.[8] Together, these studies suggest that TMIGD2 has multiple functions depending on the cell type and signaling pathways.

In summary, we have shown that the HHLA2 pathway could represents a novel immunosuppressive mechanism within the tumor microenvironment and is an attractive target for human cancer therapy. HHLA2's overexpression may be advantageous to cancer growth and survival through different mechanisms (**Fig. 1B**). Tumor-expressed HHLA2 could protect the tumor from immune surveillance via its interaction with unidentified receptors on activated T cells and other immune cells, and it may also promote angiogenesis within the microenvironment via its interaction with endothelial-expressed TMIGD2. The blockade of the B7–1/B7–2/CTLA-4 and PD-L1/PD-L2/PD-1 pathways within the B7 and CD28 families to enhance anti-tumor immunity has been exploited with therapeutic success.[9,10] Interestingly, therapies targeting HHLA2 could not only enhance anti-tumor immune responses, but may also inhibit tumor angiogenesis. Further studies are required to dissect TMIGD2's expression patterns and functions in order to develop new therapies targeting TMIGD2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Review

CellPress

New immunotherapies targeting the PD-1 pathway

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Ligands from the B7 family bind to receptors of the CD28 family, which regulate early T cell activation in lymphoid organs and control inflammation and autoimmunity in peripheral tissues. Programmed death-1 (PD-1), a member of the CD28 family, is an inhibitory receptor on T cells and is responsible for their dysfunction in infectious diseases and cancers. The complex mechanisms controlling the expression and signaling of PD-1 and programmed death ligand 1 (PD-L1) are emerging. Recently completed and ongoing clinical trials that target these molecules have shown remarkable success by generating durable clinical responses in some cancer patients. In chronic viral infections, preclinical data reveal that targeting PD-1 and its ligands can improve T cell responses and virus clearance. There is also promise in stimulating this pathway for the treatment of autoimmune and inflammatory disorders.

Expression of PD-1 and its ligands PD-L1 and PD-L2

PD-1 (CD279) is an inhibitory receptor from the CD28 family that is expressed on various immune cells including T and B lymphocytes, dendritic cells (DCs), monocytes, and macrophages [1–4]. While PD-1 is not expressed on naïve T cells, it is upregulated following T cell receptor (TCR)-mediated activation and is readily observed on both activated and exhausted T cells [5,6]. These 'exhausted' T cells have a dysfunctional phenotype and are unable to respond appropriately to stimuli. Although PD-1 has a relatively wide expression pattern, its most important role is likely as a coinhibitory receptor on T cells. Current therapeutic approaches focus on blocking the interaction of this receptor with its ligands to enhance T cell responses.

PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are both B7 family members and are currently the only known ligands for PD-1 [3,7,8]. However, their effects are not exclusively mediated through PD-1 because PD-L1 interacts with B7-1, and PD-L2 can bind to another receptor, RGMb [9,10]. Although both PD-L1 and PD-L2 bind to PD-1 and deliver coinhibitory signals to T cells, their expression patterns differ significantly. PD-L2 is expressed in relatively few cells and tissues but is upregulated on activated antigen-presenting cells (APCs) including monocytes, macrophage, and DCs [4].

PD-L1 expression is much more diverse. PD-L1 can be seen on T cells, B cells, monocytes, macrophages, and DCs, and is typically upregulated with activation. Unlike the classic B7 family members, B7-1 and B7-2, which are mainly restricted to expression on APCs, PD-L1 is expressed in several non-hematopoietic tissues including the heart, pancreas, placenta, vascular endothelium, liver, lung, and skin [2,7]. This tissue expression plays an important role in regulating immune responses in the periphery [11,12]. In addition to these normal tissues, PD-L1 is often overexpressed on cancers as a mechanism for the cancerous cells to avoid immune surveillance. It is most likely that PD-L1/L2 expression on APCs and non-hematopoietic tissue (including tumors) is the most important from a therapeutic standpoint.

Glossary

Immune-related progression-free survival: in immune checkpoint inhibitor trials on initial treatment some lesions may progress or can worsen, which by traditional standards would have been considered progressive disease when it could actually be immune-mediated eradication of disease. Hence new criteria for the classification of immunological adverse events have been proposed and PFS measured according to the new criteria.
Immunological serious adverse events: adverse events which are autoimmune in nature and could have been potentially caused by immune drugs are termed 'immunological SAE' examples, autoimmune colitis, thyroiditis, pneumonitis.
Objective response rate (ORR): the proportion of patients with tumor size reduction of a predefined amount and for a minimum time-period.
Overall survival (OS): the percentage of people in a study who are alive for a specified period of time after they were diagnosed with or started treatment for cancer.
Progression-free survival (PFS): the PFS is defined as the time from assignment in a clinical trial until either progression of the disease or death of the patient due to any cause.
Serious adverse events (SAEs): unfavorable symptoms, signs, or laboratory values which, in the view of either the investigator or sponsor, result in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions (21CFR312.32). Adverse events are graded according to the Common Terminology Criteria for Adverse Events (CTCAE) on a scale of 1 to 5 where grade 1 is a mild adverse event and grade 5 is death. In clinical trials, grade 3 or 4 SAE usually require dose adjustment or stopping the drug.

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Beginning with the observation that PD-1 knockout mice develop spontaneous autoimmunity, it has since been demonstrated in numerous studies that the PD-1/PD-L1/L2 pathway is important for T cell regulation in a variety of infectious, autoimmune, and cancer models in mice [13]. These studies largely demonstrate an important role for these molecules in regulating T cell responses; this forms the basis for the development of a new generation of targeted therapies against PD-1 and PD-L1.

In this review we begin by covering the important roles of these molecules and their mechanisms of expression and signaling. This is an exciting time to review these molecules because we are only now beginning to see patients benefiting from over two decades of basic research focused on this pathway. We review the therapeutic potential of this pathway and summarize the latest clinical trial results of drugs targeting PD-1 and PD-L1.

PD-1 signaling

Signaling through PD-1 is triggered by engagement with its known ligands, PD-L1 and PD-L2. Despite the name of the receptor, cell death is not the primary result of engagement. Instead, the primary effect of this signaling is to inhibit TCR and essential costimulatory signals (Figure 1). Upon engagement, PD-1 clusters and localizes to the TCR complex [14]. PD-1 can inhibit the phosphorylation of the TCR CD3ζ chains and Zap-70, which are early steps following TCR engagement [14–16]. Downstream activation of Ras, an enhancer of survival and proliferation, is also inhibited by PD-1 [17]. Together with the direct TCR signals, CD28 delivers costimulatory signals by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. PD-1 signaling represses this pathway by blocking PI3K activation [15]. This action begins with the phosphorylation of PD-1's intracellular immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM). The ITSM appears to be the more important of these two motifs [16,18]. The phosphorylated ITSM recruits the tyrosine phosphatase, SHP-2 [14,15]. This phosphatase leads to the inactivation of PI3K and downstream inhibition of the Akt pathway. Of note, although both PD-1 and CTLA-4 inhibit T cells, the mechanisms engaged by these two receptors are distinct [15].

The downstream signaling effects through PD-1 are numerous (Figure 1). As with other coinhibitory receptors, a decrease in T cell proliferation is seen together with a decrease in several inflammatory cytokines including

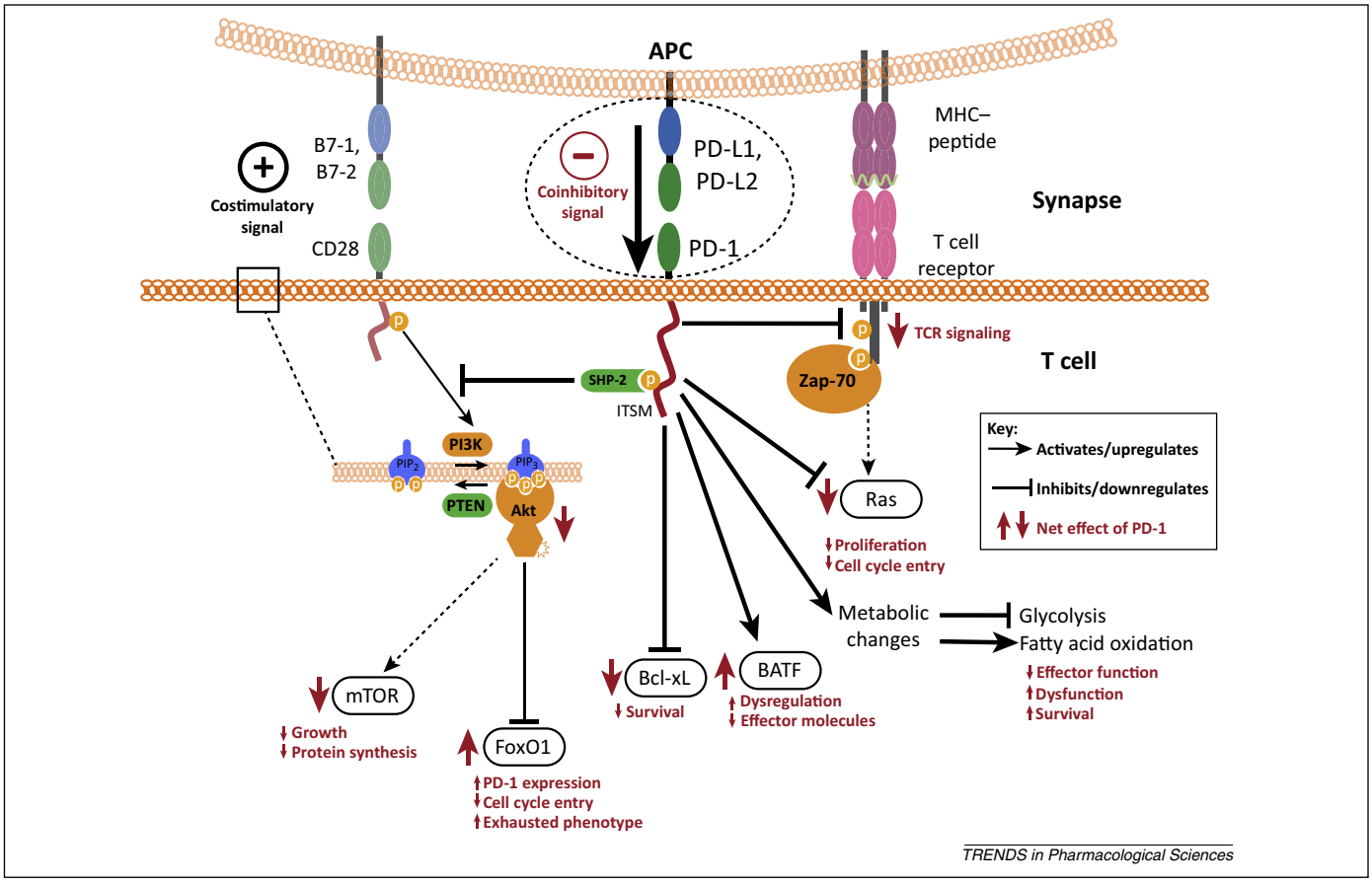

Figure 1. Programmed death-1 (PD-1) signaling. PD-1 has both an intracellular immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. SHP-2 can bind to the phosphorylated ITSM. Binding of ligands to PD-1 leads to overall inhibition of T cell receptor (TCR) signaling through inhibition of CD3ζ chain phosphorylation and Zap-70 association. PD-1 signaling causes the downregulation of both Ras and Bcl-xL which affect proliferation and cell survival, respectively. An increase in BATF can be seen which impairs the effector function of T cells. PD-1 also inhibits the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by inhibiting the activation of PI3K. This has downstream effects including downregulation of mechanistic target of rapamycin (mTOR) and an increased half-life of FoxO1. PD-1 signaling also influences cellular metabolism by inhibiting glycolysis and promoting fatty acid oxidation. Together, all these effects cause T cells to become less proliferative, lose their effector functions, and take on an exhausted and dysfunctional phenotype. The net effect of PD-1 ligation on all of these processes is shown in red, with arrow direction indicating upregulation and downregulation. Abbreviation: APC, antigen-presenting cell.

tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin 2 (IL-2) [2,3,6]. PD-1 signaling also appears to be self-reinforcing. Activation of this receptor protects the transcription factor, FoxO1, from degradation, which leads to increased expression of PD-1 [19].

More global effects are also seen on T cells. It has been shown that PD-L1 plays an important role in the differentiation of inducible regulatory T cells (iTregs) both *in vitro* and *in vivo* [20]. PD-L1 expression not only on APCs but also on other non-hematopoietic tissues may be capable of inducing Tregs. PD-1 signaling is accompanied by downregulation of phospho-Akt, mechanistic target of rapamycin (mTOR), S6, and Erk2, and by upregulation of phosphatase and tensin homolog (PTEN) [20]. Earlier work demonstrated that the Akt signaling pathway is a strong inhibitor of iTreg development, and this supports the proposed mechanism of the generation of PD-L1-induced Tregs [21].

It was also recently shown that PD-1 signaling influences the metabolism of T cells [22]. PD-1 signaling results in the inhibition of glycolysis and metabolism of amino acids while simultaneously promoting fatty acid oxidation [22]. These effects on T cell metabolism are consistent with an inhibition or reversal of effector function, and may partly explain the mechanism of impaired function seen in PD-1$^+$ T cells.

PD-1 plays an important role in exhausted T cells. It was first noted that, in chronic viral infections, PD-1 was upregulated selectively on exhausted CD8 T cells [6]. This observation has been seen in numerous chronic viral infections in both mice and humans [6,23–27]. PD-1 expression by T cells in the tumor microenvironment is also associated with an exhausted and dysfunctional phenotype [28]. Most importantly, blockade of the PD-1 signaling is able to restore CD8 T cell function and allows recovery of cytotoxic capabilities from the exhausted phenotype [29]. This treatment results in improved control of viral infection in several animal models and is the basis for future clinical trials manipulating PD-1 signaling in infectious disease.

Mechanisms controlling PD-1 expression

Considering the clinical importance of these molecules, there is great interest in understanding the mechanisms behind their expression. PD-1 is upregulated on T cells following TCR ligation [5] (Figure 2A). Cytokine signals are also important for the regulation of this molecule. Signaling through the common γ chain appears to be important, and ligands of the common γ chain, IL-2, IL-7, IL-15, and IL-21, can upregulate PD-1 expression on T cells [30].

Several more direct transcriptional mechanisms have been found as well. The transcription factor, T-bet, directly and actively represses *PD-1* gene expression [25]. After repeated antigenic stimulation, T-bet is downregulated, which leads to PD-1 expression and exhaustion. IL-6 and IL-12 (via STAT3 and STAT4, respectively) can also induce PD-1 in activated T cells through distal regulatory elements that interact with the *PD-1* gene promoter [31]. NFATc1 is a transcription factor that directly activates *PD-1* expression [31,32]. Blimp-1 inhibits *PD-1*

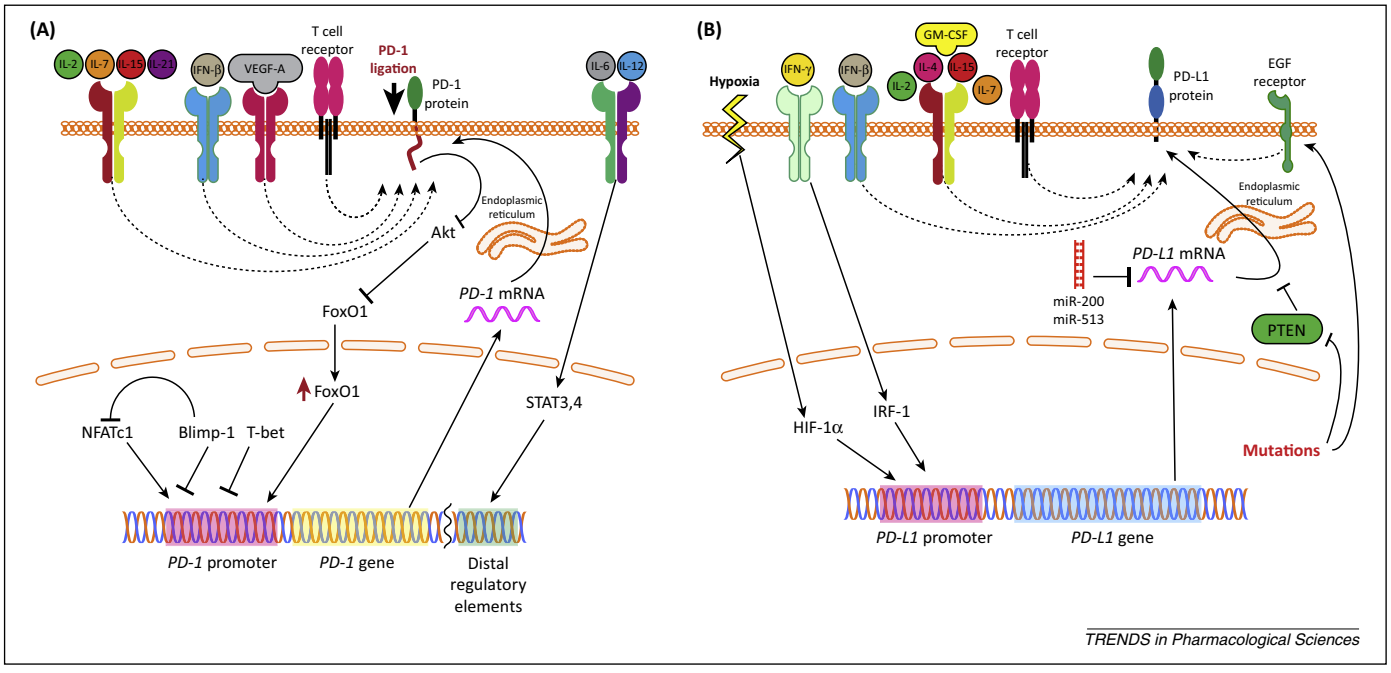

Figure 2. Regulation of programmed death-1 (PD-1) and programmed death ligand 1 (PD-L1) expression. PD-1 and its ligands are regulated by a complex network of factors. **(A)** PD-1 expression on T cells can be upregulated by numerous cytokines. Many of the common γ chain cytokines (interleukin-2, IL-7, IL-15, IL-21) can upregulate PD-1. IL-6 and IL-12, acting through signal transducer and activator of transcription 3 (STAT3) and STAT4, respectively, enhance expression of PD-1 through distal regulatory elements. Of particular relevance to the tumor microenvironment, vascular endothelial growth factor A (VEGF-A) can upregulate PD-1 through a VEGF receptor found on T cells. The nuclear factors FoxO1 and NFATc1 upregulate PD-1 through its promoter. Blimp-1 and T-bet also interact with the promoter but block its expression. Blimp-1 also functions by inhibiting NFATc1 promoter-binding. **(B)** PD-L1 expression is also regulated by numerous mechanisms. Like PD-1, several of the common γ chain cytokines upregulate it. IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also strong upregulators of both PD-L1 and PD-L2. In IFN-γ signaling, IRF-1 can bind to IFN response elements in the promoter of *PDL1*. Hypoxia can lead to upregulation of HIF-α which binds to the *PDL1* promoter and stimulates expression. Mutations of the EGFR receptor and loss of PTEN in tumors can upregulate PD-L1. Another post-transcriptional mechanism of regulation is through microRNAs. miR-200 suppression leads not only to cancer stage progression but also to simultaneous upregulation of PD-L1. miR-513 can similarly regulate PD-L1 expression in biliary epithelial cells.

expression in viral infection by not only repressing NFATc1 but also by generating suppressive chromatin changes at the *PD-1* locus [27]. Other epigenetic modifications have been described including regulation of *PD-1* by DNA methylation. Viral infection leads to a loss of this methylation in CD8 T cells which then allows transcription of *PD-1* [26,33]. This demethylation is directly related to the strength and duration of TCR signaling [26]. FoxO1 is another important transcription factor that promotes an exhausted cytotoxic T cell profile and upregulates *PD-1* [19]. FoxO1 is of particular importance because PD-1 signaling prevents FoxO1 degradation and thus defines a positive feedback loop where PD-1 signaling promotes the expression of more PD-1 [19].

PD-1 expression on T cells within the tumor microenvironment is a highly important factor in the use of immunotherapy for the treatment of cancers. PD-1 expression on T cells is predictive of response to therapies targeting this signaling pathway [34]. Beyond general T cell activation and local cytokines promoting expression of PD-1, it has been shown that vascular endothelial growth factor A (VEGF-A) can promote PD-1 expression on CD8 T cells through a VEGF receptor on these cells [35]. From all of these studies we can see that there is a complex network of many distinct mechanisms that influence the expression of PD-1.

Mechanisms regulating expression of PD-L1 and PD-L2

While PD-L1 and PD-L2 share some similarity in the molecules that induce them, there are some clear differences as well. Relatively little is known about the mechanisms regulating PD-L2 expression compared to PD-L1.

Several of the common γ chain cytokines, IL-2, IL-7, and IL-15, upregulate PD-L1 on monocytes and macrophages as well as on T cells (Figure 2B). IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4 upregulate both PD-L1 and PD-L2 on macrophages [3,4]. IL-4 and GM-CSF appear to have the most profound effect on the expression of PD-L2. Downstream IFN-γ signaling specifically results in binding of interferon regulatory factor-1(IRF-1) to the *PD-L1* gene promoter [36].

PD-L1 overexpression on tumors has also been studied. While many of the mechanisms upregulating expression may be similar to those seen in leukocytes, several tumor-specific triggers have also been identified. Loss of PTEN is a common mutation in tumors and leads to overactivation of the PI3K/Akt pathway. This mutation and the ensuing downstream signaling can lead to overexpression of PD-L1 [37]. This overexpression mechanism is largely post-transcriptionally mediated. Similarly, there is evidence that overstimulation of the epidermal growth factor receptor (EGFR) pathway, which is often found in cancers with EGFR mutations, can lead to upregulation of PD-L1 in human cancer cells [38]. Another study showed a trend toward NRAS mutations being associated with higher PD-L1 levels [39]. Non-mutagenic mechanisms have also been established. It has been shown that several important signaling pathways including the PI3K and mitogen-activated protein kinase (MAPK) pathways can modify PD-L1 expression [40]. They also showed that pharmacologically manipulating these pathways may be a possible strategy to modify PD-L1 expression in tumors. Another group has shown specific evidence that treatment of melanoma patients with MAPK inhibitors will likely be beneficial in patients whose tumors express PD-L1 and contain tumor infiltrating lymphocytes (TILs) before treatment [41]. A feature common to nearly all solid tumors is hypoxia, which can lead to induction of the transcription factor, hypoxia-inducible factor-1α (HIF-1α). HIF-1α can bind to a hypoxia response element in the *PD-L1* promoter and lead to expression of PD-L1 not only on tumor cells but also on myeloid-derived suppressor cells (MDSCs), macrophages, and DCs within the tumor microenvironment [42]. MicroRNAs also play a role in regulating tumor-expressed PD-L1. Downregulation of miR-200 in tumors leads not only to metastasis but also a simultaneous enhancement of expression of PD-L1 [43]. In other tissues, miR-513 similarly targets degradation of the *PD-L1* transcript [44].

Immunotherapy targeting PD-1 in chronic infection

Chronic infection results in a sustained high level of antigen exposure, which ultimately leads to T cell exhaustion [45]. In a mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection, blocking PD-1 and lymphocyte-activation gene (LAG-3) simultaneously reversed the exhausted phenotype and led to the clearance of viral infection [46]. T cell exhaustion is also found in chronic infections such as HIV [47], and hepatitis B and C virus (HBV, HCV) infections in humans [48,49]. Reversal of the exhausted phenotype can be achieved by blocking PD-1, and this leads to clearance of the virus.

The proof of principle of this approach was demonstrated when the CTLA-4 inhibitor, tremelimumab, was tested in a Phase I trial in hepatocellular carcinoma and chronic HCV infection. Tremelimumab (15 mg/kg IV every 90 days) was administered until cancer progression. In this study, HCV viral loads declined in most patients and there was an increase in virus-specific IFN-γ-producing lymphocytes post-treatment [50]. Nivolumab, an anti-PD-1 monoclonal antibody, was tested in IFN-refractory (n = 42) and -naïve (n = 12) patients with chronic HCV infection [51]. Patients were randomized 5:1 to receive a single infusion of nivolumab in a dose-escalation protocol or of placebo (n = 7). Five patients in the nivolumab arm had a significant reduction in HCV RNA; three achieved a >4 log reduction, two patients achieved RNA below the lower limit of quantitation, and one remained RNA-undetectable 1 year post-study. Nivolumab was well tolerated and one patient had an asymptomatic alanine transaminase (ALT) elevation. Nivolumab and anti-PD-L1 treatments are being tested in HIV patients on antiretroviral therapy to eliminate the undetectable reservoir of viral infection. These studies show that reversing T cell exhaustion can be one strategy to control chronic viral infections.

Anti-PD-1 inhibitors in cancer therapy

The success of inhibiting the central immune check point, CTLA-4, in melanoma [52,53] led to the development of peripheral checkpoint inhibitors targeting the PD-1/PD-L1 pathway. PD-1 inhibitors block the interaction of the ligands, PD-L1 and PD-L2, with T cells and increase T cell proliferation and function [54]. The PD-1 inhibitors

currently in clinical trials are nivolumab (MDX-1106/BMS-936558, Bristol Meyers Squibb), pembrolizumab (MK-3475, Merck) and pidilizumab (CT-011, Cure Tech); these have some differences.

Nivolumab and pembrolizumab are fully human IgG4 and humanized IgG4 monoclonal antibodies (mAbs), respectively. Unlike the IgG1 and IgG3 subtypes, IgG4 has markedly decreased antibody dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity, which prevents depletion of activated T cells [55]. Below we present an overview of selected trials of PD-1 inhibitors in solid tumors and hematological malignancies (Table 1).

Melanoma

Melanoma is a known immunogenic tumor, and TILs in melanoma have been shown to colocalize with melanocytes expressing PD-L1. This interaction of T cells with tumor-expressed PD-L1 contributes to immune evasion in melanoma [56]. Ipilimumab (a monoclonal antibody against CTLA-4) demonstrated an overall survival (OS; see Glossary) benefit in two Phase III trials in metastatic melanoma [53,57]. However, approximately only 20% of patients with metastatic melanoma survive after 3 years even after ipilimumab, leaving marked room for improvement.

In a Phase I study of refractory melanoma patients, nivolumab had an objective response rate (ORR) of 31%

Table 1. Clinical trials of antibodies to PD-1[a]

Drug	NCT	Phase	Design	Population	*n*	Key findings/conclusions
Melanoma						
Nivolumab	NCT00730639	I	Dose finding and dose expansion	Advanced melanoma	107	OS = 43% at 2 years which compares favorably with historical population
	NCT01176461	I	Nivolumab +/- peptide vaccine (NY-ESO-1, gp100, MART-1)	Advance melanoma, Ipilimumab naïve and refractory	90	ORR = 25.1%. No difference in response between Ipilimumab naïve and refractory or addition of vaccine to nivolumab. PD-L1 negative patients also responded.
	NCT01721772	III	Nivolumab versus dacarbazine	Metastatic melanoma without BRAF mutation	418	Significantly better 1 yr OS in nivolumab arm (72.9% vs 42.1%). Relatively well tolerated
	NCT01721746	III	Nivolumab versus investigators choice	Metastatic melanoma after CTLA-4 or BRAF inhibitor therapy	370	Results in 167 patients showed higher ORR in nivolumab arm and durable tumor regression as well
Pembrolizumab	NCT01295827	I	Dose finding and dose expansion	Advanced melanoma including CTLA4 treated patients	135	ORR = 38%. No difference in response between ipilimumab naïve and refractory. Acceptable safety profile and slightly better responses in the higher dose 10 mg/kg arm
	NCT01295827	I	Nivolumab 2 mg/kg versus 10 mg/kg	Advanced melanoma whose disease progressed on ipilimumab	173	ORR = 26% after ipilimumab therapy. No difference between the two drug doses.
Pidilizumab	NCT01435369	II	Pidilizumab 1.5 versus 6 mg/kg	Advanced melanoma	103	Low response rate of 5.9%
Non-small cell lung cancer						
Nivolumab	NCT00730639	I	Dose finding and dose escalation	Advanced malignancies	296	ORR = 18.4% in NSCLC cohort. Low rate of Grade 3 SAE = 14%
	NCT01454102	I	Nivolumab + erlotinib	Stage IIB/IV NSCLC in EGFR mutated patients naïve or post progression	21	ORR = 19% with an acceptable safety profile
	NCT01642004	III	Open-label randomized nivolumab vs docetaxel	Metastatic squamous cell lung cancer after 1 line of platinum based therapy	272	Superior OS in the nivolumab arm at 1 year (9.2 vs 6 mos.) with durable responses
Pembrolizumab	NCT01295827	I	Pembrolizumab at 2 mg/kg or 10 mg/kg	Advanced NSCLC	282	ORR = 21%, PD-L1$^+$ tumors had higher response rates than negative tumors
Genitourinary malignancies						
Nivolumab	NCT01354431	II	Nivolumab at 0.3 versus 2 versus 10 mg/kg doses	Previously treated RCC with VEGF inhibitors	168	ORR = 21% across all 3 arms with a tolerable safety profile. No dose response relationship was response
Pembrolizumab	NCT01848834	Ib	Dose-finding study	PD-L1+ >1% and advanced urothelial cancer	33	ORR = 24%, with CR in 10% with an acceptable safety profile
Other cancers						
Nivolumab	NCT01592370	I	Dose escalation and dose expansion	Relapsed, refractory Hodgkin lymphoma	23	ORR = 87% with a PFS at 24 weeks of 86% with an acceptable safety profile.
Nivolumab	NCT01876511	II	Pembrolizumab 10 mg/kg q2 weeks	Advanced malignancies with or without mismatch deficiency	41	IrPFS = 78% versus 11% for mismatch deficient versus proficient colorectal cancers

[a]CR, Complete response; irPFS, immune related progression-free survival; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival, PFS, progression-free survival; SAE, serious adverse events.

with grade 3/4 serious adverse events (SAEs) in 22% of patients [58]. These results demonstrated both efficacy and acceptable safety of nivolumab in melanoma patients. In another Phase I study, prior treatment with ipilimumab or the addition of a peptide vaccine to melanoma antigens did not affect responses to nivolumab [59]. These results support basic research data showing that the immune checkpoints, CTLA-4 and PD-1, signal through mechanistically distinct pathways [15]. A randomized Phase III trial ($n = 418$) compared nivolumab at 3 mg/kg every 2 weeks ($n = 210$) with dacarbazine (chemotherapy) in BRAF-negative previously untreated metastatic melanoma [60]. The ORR (40% vs 13.9%), progression-free survival (PFS) (5.1 vs 2.2 months), and OS at 1 year (72.9% vs 42.1%) were significantly better in the nivolumab arm compared to dacarbazine. Moreover, grade 3/4 adverse effects were slightly reduced in the nivolumab arm (11.7 vs 17.6%) and immunological adverse events occurred in 1–2% of patients. In another open-label Phase III study, patients with metastatic melanoma who progressed on ipilimumab were randomized to nivolumab or to the investigators' choice of chemotherapy. The ORR was higher in the nivolumab arm (32% vs 11%), with durable tumor regression in responders [61]. Based on these results, nivolumab received FDA approval in December 2014 for patients with melanoma who were previously treated with ipilimumab or a BRAF inhibitor. Recently in a Phase I study the combination of two immune checkpoint inhibitors, ipilimumab and nivolumab, was safe and produced superior responses than ipilimumab alone for the upfront treatment of metastatic melanoma [62].

Pembrolizumab was studied in a dose-escalation study with a dose range of 1–10 mg/kg in 135 patients with refractory melanoma, some of whom received prior ipilimumab treatment [63]. The ORR was 38% and grade 3/4 adverse events were present in 13% of patients; there was no difference between ipilimumab-naïve and refractory patients. Based on these safety data KEYNOTE-001, an open-label trial, tested pembrolizumab in two doses at 2 mg/kg or 10 mg/kg after progression on ipilimumab and BRAF or MEK inhibitors, in BRAF-mutant tumors [64]. 173 patients with metastatic melanoma received pembrolizumab and the ORR was 26% in both groups and grade 3 to 4 SAEs were reported as 12%. The safety and efficacy of the 2 mg/kg and the 10 mg/kg doses were comparable with no significant benefit of the increased dose. Pembrolizumab was granted breakthrough status by the FDA for the treatment of ipilimumab- or BRAF inhibitor-refractory metastatic melanoma patients.

Non-small cell lung cancer

In non-small cell lung cancer (NSCLC), PD-1 is expressed in 35% of TILs and PD-L1 is expressed 20-25% of lung cancer specimens. Constitutive oncogenic signaling through the PI3K or EGFR pathway [37,38] or cytokine secretion by lymphocytes leads to activation of the PD-1/PD-L1 pathway in NSCLC [65].

Nivolumab was first tested in a dose-escalation Phase I trial of refractory malignancies of whom 129 had metastatic NSCLC [66,67]. The ORR in NSCLC was 18% with 33% of squamous and 12% of non-squamous cancers responding. The OS at 1 year was 42% and the median duration of response was 74 weeks and a sustained response of >24 weeks was seen in 57% of patients. Grade 3/4 toxicities were present in only 6% of patients and pneumonitis occurred in 7% of patients. Pneumonitis is a concern in these patients as they already can have poor lung reserve. Nivolumab was approved by the FDA for treatment of squamous NSCLC after progression on a platinum-based chemotherapy regimen. This approval was based on the results of an open-label, multicenter, randomized trial of 272 patients with metastatic squamous NSCLC who were randomized to docetaxel or nivolumab at 3 mg/kg every 2 weeks [68]. There was a significant improvement in median OS of 9.2 (nivolumab) versus 6 months (docetaxel) seen for patients receiving nivolumab. This represents a significant improvement for patients with squamous NSCLC whose treatment options are limited.

Pembrolizumab, in a pooled analysis of 262 relapsed NSCLC patients (KEYNOTE-001), had an ORR of 21% as a single agent, and results were similar in patients with squamous or non-squamous histology [69]. In patients with strong PD-L1 expression (>50%) the ORR was 39% and 16% in weak/negative expression suggesting that PD-L1 alone cannot be used as a biomarker to select patients. The FDA granted breakthrough status for pembrolizumab in lung cancer in October 2014.

Genitourinary malignancies

In renal cell cancer, increased TILs along with high PD-L1 expression in the initial biopsy is associated with shorter survival in patients treated with tyrosine kinase inhibitors (TKIs) for metastatic disease [70]. Similarly, high PD-L1 expression is associated with failure of response to Bacillus Calmette Guerin (BCG) for localized bladder cancer by neutralizing the T cell response to BCG immunotherapy [71]. These data suggest that the PD-1 axis contributes to resistant disease in urothelial malignancies.

Nivolumab was tested in a dose-escalation Phase I trial of patients with refractory malignancies of whom 33 had metastatic renal cell cancer with an ORR of 27% [66]. In a Phase II trial of 168 clear cell renal cell cancer (RCC) patients, nivolumab was tested at three doses of 0.3, 2, or 10 mg/kg and the median OS was 18.2, 25.5, and 24.7 months, respectively, which was higher than the historical OS rates of 11-16.5 months in this cancer [72]. As a result, a Phase III randomized study evaluating nivolumab and everolimus as a second-line therapy for metastatic RCC is underway.

Pembrolizumab was similarly tested in the KEYNOTE-012 study in 33 patients with metastatic urothelial cancer at 10 mg/kg and the ORR was 24.1% with a median OS of 9.3 months [73]. These studies show favorable efficacy and acceptable safety of PD-1 inhibitors in bladder and renal cancers and are highly likely to move forward in clinical trials.

Other tumors: Colon cancer, Hodgkin lymphoma

Hodgkin lymphoma is a B cell tumor in which the PD-1/PD-L1 axis is activated by JAK signaling and chromosomal amplifications in the 9p24.1 region which codes for the PD-L1/PD-L2 ligands. In an ongoing Phase I study of

Table 2. Clinical trials of antibodies to PD-L1[a]

Drug	NCT	Phase	Design	Population	*n*	Key findings/conclusions
Unselected						
BMS-936559	NCT00729664	I	Dose escalation and dose expansion	Advanced refractory malignancies	207	ORR: 6–17% and PFS: 12–41% at 24 weeks. Acceptable safety profile. Positive signal in melanoma, renal cell cancer, NSCLC and ovarian cancer with durable responses
MPDL3280A	NCT01375842	I	Dose escalation and dose expansion	Advanced solid tumor cancers	171	ORR = 21% with an acceptable safety profile. PD-L1$^+$ status resulted in higher responses. No pneumonitis related deaths
Genitourinary malignancies						
MPDL3280A	NCT01375842	I	Dose escalation and dose expansion	Metastatic urothelial bladder cancer	31	ORR = 50% with treatment response showing increase in CD8$^+$Ki67$^+$ T cells
Non-small cell lung cancer						
MEDI4736	NCT01693562	I	Dose escalation and dose expansion	Advanced NSCLC	13	ORR = 5/13 patients responded. No grade 3 pneumonitis observed

[a]CR, complete response; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall Survival; PFS, progression-free survival; SAE, serious adverse events.

23 patients with relapsed Hodgkin lymphoma, the ORR was 87% with a 17% complete response rate [74]. A recent Phase 2 study in colon cancer showed that immune-related progression-free survival rates were superior in mismatch-deficient compared to mismatch-proficient colon cancers (78 vs 11%) [46]. These studies show that these agents are likely to be effective across a wide variety of malignancies.

Anti-PD-L1 inhibitors in cancer therapy

Antibodies against PD-L1 act by blocking the interaction of PD-L1 with PD-1 but do not block the interaction of PD-1 with PD-L2. This may help to decrease toxicity since the PD-1/PD-L2 pathway still plays a role in peripheral tolerance. The three therapeutic monoclonal antibodies against PD-L1 are BMS-986559 (MDX-1105), MPDL3280A, and MEDI4736 and are in various phases of clinical trials. We briefly discuss here the clinical trials with these agents (Table 2).

BMS-936559 was first tested in a multicenter Phase I dose-escalation trial (0.3 to 10 mg/kg every 14 days in 6 week cycles) in patients with refractory malignancies [75], including melanoma, NSCLC, colorectal, renal cell, ovarian, pancreatic, and breast cancer (n = 207). The median duration of therapy was 12 weeks (range, 2 to 111) and SAEs occurred in 9% of patients. Patients with melanoma (9/52), renal cell (2/17), NSCLC (5/49), and ovarian cancer (1/17) had responses, and half of these responses were sustained for more than 1 year. It is currently not being developed as a clinical agent in malignancies despite its initial promise.

MPDL3280A is a bioengineered anti-PD-L1 antibody with minimal ADCC and CDC activity. In a Phase I dose-escalation trial of advanced solid tumors, no maximum tolerated dose (MTD) was defined at escalating doses [76]. The ORR was 21%, 24 week PFS was 44% and patients with PD-L1-positive tumors had a higher ORR (39%) than those with negative tumors (13%). Interestingly, there was no grade 3–5 pneumonitis or diarrhea in this small study, suggesting that the PD-L2 pathway (not inhibited by the anti-PD-L1 antibodies) could be important in minimizing toxicity. Further, in a Phase I study of urothelial cancer MPDL3280A showed significant activity (ORR 26%) with a good duration of response [77]. Based on these data, MPDL3208A received breakthrough status for bladder and NSCLC.

MEDI4736 is an IgG1 monoclonal antibody against PD-L1 that is being tested in an ongoing Phase I trial in NSCLC patients and shows preliminary clinical activity with a favorable toxicity profile [78]. Based on the Phase I results, a Phase III trial in patients with locally advanced NSCLC is being planned.

Concluding remarks

Under physiological conditions the PD-1 pathway is important for maintaining peripheral immune tolerance. This pathway represents one of the many redundant pathways to prevent inappropriate immune responses. Such redundant coinhibitory pathways are exploited by tumors and chronic viral infections to cause T cell exhaustion,

Box 1. Outstanding questions

Biomarkers of response

- Although therapies targeting PD-1 and PD-L1 are highly effective when they work, their current response rates leave much to be desired. Understanding what factors determine if a patient will respond is a crucial next step to advancing the use of these therapies. Predictors of response to immune checkpoint inhibitors could be related to tumor-associated factors or host factors. Tumor expression of PD-L1, specific mutations in the tumor, and the presence of tumor antigen-specific T cells are all examples of potential biomarkers currently being assessed. For example, PD-L1 expression will likely be a useful biomarker because patients with PD-L1 expression appear to have a higher response to anti-PD-1 therapy than those without [34]. In a retrospective analysis, NRAS mutant melanoma had a higher response rate to anti-PD-1 therapy [39]. CD8$^+$, PD-1$^+$, and PD-L1$^+$ cells in the tumor margins correlate with response to anti-PD-1 therapy in melanoma [34]. A highly restricted TCR repertoire also correlated positively with responses. In NSCLC, higher nonsynonymous mutational burden leading to increased neoantigens was associated with better responses to pembrolizumab [79]. Similarly, mismatch repair-deficient colon cancers which have a higher somatic mutational burden responded better to anti-PD-1 therapy [46]. In summary, there are clearly a variety of factors that control whether a patient will respond well to these therapies. Current and future work will address what these markers are and their relative importance. This work will be important not only for guiding therapeutic choices in patient treatment but also for finding strategies to enhance responses in patients treated with these drugs.

which results in tumor immune evasion and decreased viral clearance. Recent therapeutic advances targeting this pathway have met with good success in human cancers. More importantly, these treatments can provide durable responses. It remains to be seen whether combinatorial approaches with radiation, chemotherapy, other coinhibitory antibodies, or vaccines can improve the response rate in cancers. Predictive biomarkers need to be developed to identify short- and long-term responders to immunotherapy (Box 1). Different cancers may result in different mechanisms of PD-1/PD-L1 expression, and hence a single biomarker may not be useful across all tumor types. Tumor-related factors include specific oncogenic pathway activations, mutational burden, and PD-L1 expression, while host factors could be the presence of prior infections or vaccinations. Bioinformatics and immunogenetic approaches will be necessary to identify relevant tumor-associated antigens to which cytotoxic T cells respond or maintain response after immune checkpoint inhibitors.

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Co-stimulate or Co-inhibit Regulatory T Cells, Which Side to Go?

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ABSTRACT

Co-stimulatory and co-inhibitory molecules direct the "second signal," which largely determines the outcome of the "first signal" generated by the interaction of T cell receptor (TCR) with cognate MHC–peptide complex. The co-stimulatory and co-inhibitory signals are key mechanistic contributors to the regulation of adaptive immunity, especially the T cell–mediated immune response. Regulatory T cells (Tregs) are a special population of T cells, which unlike other T cells function as "attenuators" to suppress T cell immunity. Dysregulation of either the "second signal" or Tregs leads to an unbalanced immune system, which can result in a range of immune-related disorders, including autoimmune diseases, chronic infections, and tumors. In contrast, precise manipulation of these two systems offers tremendous clinical opportunities to treat these same diseases. Co-stimulatory and co-inhibitory molecules modulate immunity at molecular level, whereas Tregs delicately control the immune response at cellular level. Accumulating evidence has demonstrated that these two regulatory strategies converge and synergize with each other. This review discusses recent progress on the roles of co-stimulatory and co-inhibitory signals in the context of Tregs.

KEYWORDS

Co-inhibition; co-stimulation; immunotherapy; Treg

Introduction

Co-stimulation and co-inhibition

Co-stimulatory and co-inhibitory molecules are pivotal cell-surface proteins, largely composed of members of the immunoglobulin superfamily (IgSF) and tumor necrosis factor/receptor superfamily (TNFSF/TNFRSF) (Chen & Flies, 2013). These molecules are vital for T cell activation and subsequent immune responses, as they provide the secondary signal that determines the course, duration, and extent of the response following the initial signal provided by engagement of the T cell receptor (TCR) and the major histocompatibility complex (MHC)–peptide complex. The co-stimulatory receptors transduce positive signals to facilitate or amplify the adaptive immune response, whereas the co-inhibitory receptors produce negative signals to attenuate the T cell response (Zang & Allison, 2007).

The CD28:B7 family members are among the most extensively studied co-stimulatory and co-inhibitory molecules. The CD28:B7 family belongs to the IgSF, the members of

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which all share similar overall structural features, with each Ig domain formed by two mixed beta-sheets (Chattopadhyay et al., 2009). The CD28:B7 family contains the first identified co-stimulatory receptor CD28, co-inhibitory receptor CTLA-4, and their joint ligands B7-1 and B7-2 (Zang & Allison, 2007). Activation of the CD28 receptor by its ligands, or agonistic antibodies, was shown to prevent T cell energy induction and promote T cell proliferation and cytokine IL-2 production, thus establishing CD28 as a central co-stimulatory receptor (Gimmi et al., 1991; Harding et al., 1992; Koulova et al., 1991; Linsley et al., 1991). In contrast to CD28, which is constitutively expressed on T cell surface and enhance T cell activity, CTLA-4 is induced following T cell activation and serves as a co-inhibitory receptor to suppress T cell response (Rudd et al., 2009). The CD28/CTLA-4 and B7-1/B7-2 pathways were the first characterized co-stimulatory and co-inhibitory pathways, and have been under intense study since their discovery. PD-1 and PD-L1/PD-L2 pathways were later reported to be another co-inhibitory pathways to inhibit the T cell–mediated immunity (Freeman et al., 2000).

Working in concert with the CD28:B7 family of the IgSF, additional co-stimulatory and co-inhibitory molecules are represented by members of the TNFSF/TNFRSF. Most of the TNF ligands form a homotrimeric assembly, with each monomer adopting the typical "jelly-roll" fold involving two parallel β-sheets. TNF receptors possess ectodomains characterized by varying numbers of tandemly linked cysteine-rich domains (CRDs) (Chattopadhyay et al., 2009). In most circumstances, one TNF ligand is able to bind three TNF receptors through the grooves between each protomer (Chattopadhyay et al., 2009). The engagement of TNF receptors with TNF ligands leads to the trimerization, in some cases dimerization, of TNF receptors and activates the intracellular signal transduction pathways involving the assembly of intracellular scaffolding and signaling complexes.

The importance of co-stimulatory and co-inhibitory molecules in regulating the immune system has been demonstrated by the successful development and clinical application of drugs targeting these molecules. Ipilimumab (Yervoy, Bristol-Myers Squibb, USA) is an FDA-approved function-blocking monoclonal antibody (mAb) that specifically targets CTLA-4 to inhibit the associated co-inhibitory signal, resulting in a systemic enhancement of T cell activity. Ipilimumab represents the first clinical treatment to significantly prolong survival in late-stage melanoma cancer patients and marks a milestone for cancer immunotherapy (Chodon et al., 2015; Zang and Allison, 2007). Selective inhibition of the PD-1/PD-L1 inhibitory pathway by mAbs has also resulted in two FDA-approved drugs pembrolizumab (Keytruda, Merck & Co., USA) and nivolumab (Opdivo, Bristol-Myers Squibb, USA) to treat cancers (Chinai et al., 2015).

Despite the enormous success on targeting these proteins to treat immune-related diseases, it has been gradually realized that the outcomes of these co-stimulatory and co-inhibitory molecules are not as straightforward as what they were originally discovered. Given the immense diversity of the cellular expression, structures, and their interaction networks, these molecules may have totally different outcomes on the adaptive immunity. For example, expression of the co-stimulatory receptor CD28 on conventional effector T cells (Teffs) upregulates immune response. However, expression of CD28 on a special suppressive T cell population, termed regulatory T cells (Tregs), promotes immune inhibition and grants some levels of immune tolerance. As more of the co-stimulatory and co-inhibitory molecules are emerging, more thorough understanding of how these

molecules affect the immunity, especially in the context of Tregs, is required for the precise manipulation of the immune system through co-stimulation and co-inhibition.

Tregs

Tregs are a T cell subpopulation produced by the normal immune system, which provide suppressive signals to prevent overly aggressive immune responses. Tregs naturally arise within the thymus as a functionally mature T cell lineage and can also be induced in the periphery from naive T cells (Sakaguchi et al., 2008). Both thymus-derived natural Tregs (tTregs) and peripherally induced Tregs (pTregs) participate in controlling the magnitude of the immunity. Depletion of Tregs can lead to the development of a range of autoimmune conditions, including colitis, which possibly result from lack of control of bacteria-driven inflammatory responses in the mucosal system (Singh et al., 2001). Conversely, elimination or reduction of Tregs can overcome the immunosuppressive mechanisms utilized by tumors or chronically infectious microbes to evade the host immune system, and provides a strategy for the eradication of tumors or microbes (Belkaid & Rouse, 2005; Wollenberg et al., 2011). Neonatal thymectomy experiments in mice cause autoimmune disease, demonstrating thymus-derived tTregs are key to immune tolerance, and also demonstrate that peripheral pTregs are not sufficient to suppress auto-reactive immunity (Asano et al., 1996; Bonomo et al., 1995; Sakaguchi et al., 1995). However, peripheral pTregs do make important contributions to controlling autoimmune responses (Haribhai et al., 2011; Josefowicz et al., 2012; Samstein et al., 2012), as pTreg deficiency is sufficient to evoke T cell–mediated autoimmune conditions (Yadav et al., 2013). In contrast, enrichment of pTregs in mice ameliorates allergy, builds immunological tolerance to transplanted organs, and enhances feto-maternal tolerance (Sakaguchi, 2005).

Tregs are characterized by the expression of cell-surface receptor CD25 and transcription factor Foxp3. CD25 is the α chain of the heterotrimeric IL-2 receptor complex, which captures IL-2 with high affinity. CD25 is critical for Treg function, as mice deficient in CD25 develop lymphoproliferative autoimmune disease and are hyperreactive to commensal microbiota, with a phenotype resembling that of pTreg knockout mice (Sakaguchi et al., 2008). The transcription factor Foxp3 programs the development and function of Tregs. In humans, mutations in the FOXP3 gene results in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett et al., 2001). Similarly, Foxp3-null mice or the mouse strain Scurfy, which is defective in Foxp3 gene, develop deleterious hyperreactive immunological phenotypes resembling IPEX (Fontenot et al., 2003). Overexpression of Foxp3 in transgenic mice increases the number of Tregs and delays the catastrophic disease in CTLA-4 $^{-/-}$ mice (Khattri et al., 2003). Ectopic expression of Foxp3 in naive T cells upregulates the expression of CD25, the co-stimulatory molecule GITR (glucocorticoid-induced tumor necrosis factor receptor related protein) and co-inhibitory molecule CTLA-4, and programs the expression of other Treg functional molecules (Fontenot et al., 2003).

Several mechanisms have been proposed for Treg-mediated suppression. For example, Tregs may secrete immunosuppressive cytokines and absorb cytokines necessary for other T cells to proliferate and function. TGF-β secreted by Tregs can mediate suppression and program the Teffs to be more susceptible to suppression (von Boehmer, 2005).

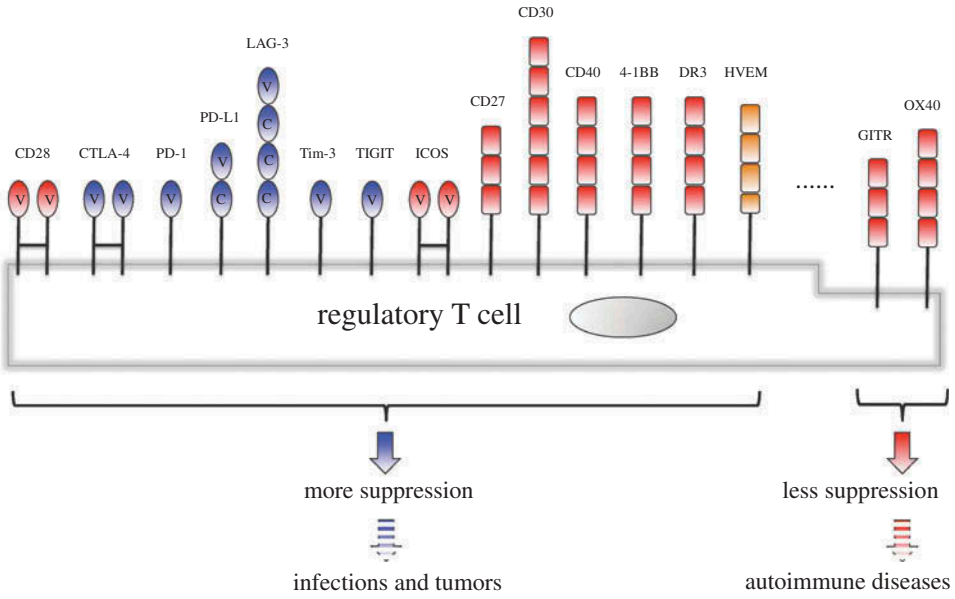

Figure 1. The co-stimulatory (red) and co-inhibitory (blue) molecules are classified as two groups based on their impact on Tregs. The left side shows the group of molecules that can enhance the suppression function of Tregs after stimulation. The right side shows the group of molecules that can impair the suppression function of Tregs after stimulation. Members of IgSF are represented as cognate numbers of Ig domains (*ovals*). "V" and "C" stand for IgV and IgC domains, respectively. Members of TNFRSF are represented as cognate numbers of CRDs (*rectangles*). HVEM is colored as orange as it can be either co-stimulatory or co-inhibitory, depending on different engaging ligands.

Competitive consumption of cytokines by Tregs, including IL-2, deprives Teffs of survival cytokines and induces apoptosis of these Teffs (Pandiyan et al., 2007). Tregs can also modify the function of, or even kill, antigen-presenting cells (APCs) in a direct cell contact–dependent manner (Sakaguchi et al., 2008). The co-stimulatory and co-inhibitory molecules expressed by both Tregs and APCs play essential roles in the development and suppressive functions of Tregs at multiple steps (Bour-Jordan & Bluestone, 2009). In this review, we discuss the roles of several co-stimulatory and co-inhibitory molecules in different aspects of Treg function (Figure 1) and explore the potential of cancer immunotherapies targeting co-stimulatory, co-inhibitory signal, and Tregs.

CD28

CD28, the prototypical co-stimulatory receptor, is constitutively expressed on almost all human $CD4^+$ T cells and on about half of human $CD8^+$ T cells, whereas it is expressed on almost all matured $CD4^+$ and $CD8^+$ T cells in mouse (Acuto & Michel, 2003). CD28 exists as a disulfide-linked homodimer on the cell surface, with each monomer composed of one single immunoglobulin variable (IgV) domain. The CD28 ligands, B7-1 and B7-2, are each composed of a membrane distal IgV domain and a membrane proximal immunoglobulin

constant (IgC) domain (Chattopadhyay et al., 2009). The interactions of CD28 with B7-1 and B7-2, involving their respective IgV domains, are involved in a wide range of T cell functions, including T cell proliferation, cytokine production, survival, and T cell–dependent antibody responses (Lenschow et al., 1996; Salomon & Bluestone, 2001). It seemed with clear evidence that CD28 and B7-1/B7-2 pathway is the most prominent driving force of positive immune response. However, surprisingly, CD28 or B7-1/B7-2 knockout mice, whereas in non-autoimmune backgrounds and many antigen-induced autoimmune backgrounds, exhibited significant immune-deficient phenotypes, and exacerbated the autoimmune conditions when bred into the non-obese diabetic (NOD) background (Bour-Jordan and Bluestone, 2009; Lenschow et al., 1996; Salomon et al., 2000). In combination with the vast Treg literature, these results for the first time suggested a role for CD28 in T cell suppressor function. It is now well established that though CD28 transduces co-stimulatory signal to Teffs, it is also required for the homeostasis and function of the suppressing Tregs.

In NOD mice with either CD28 or B7-1/B7-2 knockout, the incidence and progression of spontaneous autoimmune diabetes were enhanced. Moreover, these mice developed other autoimmune disorders such as autoimmune exocrine pancreatitis, and displayed higher degree of lymphocyte penetration and more severe diabetes compared with the controls (Lenschow et al., 1996; Meagher et al., 2008; Salomon et al., 2000). Further assessment of B7 knockout NOD mice revealed that the immunoregulatory $CD4^+CD25^+$ Tregs were absent. Injection of the wildtype $CD4^+CD25^+$ Tregs into CD28 knockout NOD mice restored the control of diabetic disease (Salomon et al., 2000; Tang et al., 2004). Additionally, blockade of CD28:B7 pathway dramatically decreases the Tregs population in all other normal and autoimmune strains (Bour-Jordan and Bluestone, 2009; Gogishvili et al., 2013; Sansom and Walker, 2006). Disruption of CD28 signaling using CTLA-4-Ig or antagonistic mAbs against B7 ligands induced a rapid decrease in the number of $CD4^+CD25^+$ Tregs within 9 days of treatment (Salomon et al., 2000). The dramatic decrease on Tregs after treatment is similar to that observed in thymectomized mice. However, thymectomized mice did not affect the Tregs within 10 days, suggesting that impairment of the CD28:B7 pathway has a direct impact on pTregs, and the CD28:B7 pathway is required for the maintenance of pTregs (Tang et al., 2003). Furthermore, transplanted Tregs exhibited similar decreases when B7-specific blocking reagents were utilized (Bour-Jordan and Bluestone, 2009). These results suggest that the CD28:B7 pathway is critical for the homeostasis of both thymus-derived tTregs and peripheral pTregs, and may leverage the threshold of the autoimmune diseases by modulating the homeostasis of Tregs.

CD28 acts in concert with the signal provided by TCR engagement to promote T cell activation and proliferation, largely by accelerating T cell division, as well as boosting cytokine production (Gett and Hodgkin, 2000; Sansom and Walker, 2006; Thompson et al., 1989). While CD28-associated signaling promotes proliferation of both Teffs and Tregs *in vitro, in vivo* studies suggest CD28 has a more profound effect on Treg proliferation (Hori et al., 2002). Several other experiments produced consistent results, which all demonstrated the rapid proliferation of Tregs *in vivo* (Fisson et al., 2003; Tang et al., 2003; Walker et al., 2003). Abrogation of CD28:B7 engagement by antagonistic anti-B7 mAbs or in B7 ligand knockout mice recipients completely prevented Treg expansion *in vivo* in a transfer model (Tang et al., 2003). Conversely, a "superagonistic" anti-CD28 mAb

preferentially expanded Tregs over other T cell subsets *in vivo*, resulting in a 20-fold Tregs expansion within 3 days after a single mAb treatment (Lin & Hünig, 2003). Administration of very low dosages of CD28 superagonist to rats dramatically enhanced the proliferation of suppressive Tregs; but, not the other T cells, and afforded protection from experimental autoimmune encephalomyelitis (EAE) (Beyersdorf et al., 2005). However, intravenous administration of agonistic anti-CD28 mAbs (working name TGN1412) even at sub-clinical dose induced rapid cytokine storms and lead to catastrophic systemic organ failures in patients of a phase 1 clinical trial (Suntharalingam et al., 2006).

CD28 enhances the survival of T cells by activating phosphatidylinositol 3-kinase (PI3K) and subsequent AKT kinase (Sansom & Walker, 2006). CD28 activation also renders T cells more resistant to apoptosis by upregulation of the prosurvival protein Bcl-x_L (Okkenhaug et al., 2001; Wu et al., 2005). As CD28 induces IL-2 secretion, CD28 may indirectly promote Treg survival by upregulating IL-2 production (Bour-Jordan and Bluestone, 2009). The role of CD28 in Treg functions has been rarely reported, largely because CD28 is required for the homeostasis of Tregs. Several recent studies using Tregs-specific *Cd28* conditional knockout mice revealed a cell-intrinsic function for CD28 in Tregs. Although the *Cd28* conditional knockout mice presented normal numbers of $Foxp3^+$ cells, the animals still developed severe autoimmune conditions, indicating CD28 is indispensable for the immunoregulatory function of Tregs (Zhang et al., 2013, 2015).

CTLA-4

CTLA-4, the co-inhibitory counterpart of CD28, also engages the B7-1 and B7-2 ligands, albeit with 10- and 100-fold higher affinities toward B7-1 and B7-2, respectively (Collins et al., 2002). Like CD28, CTLA-4 is composed of a single extracellular IgV domain followed by a stalk region, a transmembrane domain, and a cytoplasmic tail. Two CTLA-4 extracellular stalk regions share a disulfide bond, which brings together two CTLA-4 to form a covalently linked homodimer (Chattopadhyay et al., 2009). In contrast to CD28, CTLA-4 expression is induced subsequent to T cell activation (Teft et al., 2006). Tregs are notable exceptions as they constitutively express CTLA-4 (Takahashi et al., 2000). The transcription factor Foxp3 has been demonstrated to upregulate CTLA-4 expression on Tregs (Sansom and Walker, 2006). Evidence for the convergence of CTLA-4 and Treg-mediated tolerance comes from the CTLA-4 knockout mice and Foxp3 knockout mice. Deficiency of CTLA-4 or Foxp3 elicits similar catastrophic autoimmune phenotypes, suggesting potential links between the CTLA-4 pathway and Treg function (Walker, 2013).

Initial reports suggested that mAb-mediated blockade of CTLA-4 resulted in loss of the suppressive functions of Tregs (Read, et al., 2000; Takahashi et al., 2000). Another possibility was that the observations were the consequence of augmented conventional T cell activity (Tconvs) due to loss of inhibitory CTLA-4 signaling (Thornton et al., 2004). In several *in vitro* Treg suppression assays, absence of the CTLA-4 signal also abrogated Treg function (Tai et al., 2012; Wing et al., 2008). The most compelling evidence in support of a role for CTLA-4 in Treg function comes from *in vivo* experiments, in which the CTLA-4 signal was specifically manipulated in Tregs. In a T cell–mediated colitis

model, administration of wildtype $CD4^+CD25^+$ Tregs was able to suppress the colitis, whereas administrations of either B7-1/B7-2/CTLA-4 knockout $CD4^+CD25^+$ Tregs or wildtype $CD4^+CD25^+$ Tregs in combination with antagonistic anti-CTLA-4 mAbs did not protect the mice from colitis (Read et al., 2006). Specific deletion of the CTLA-4 gene in Tregs impaired Treg-mediated suppression and resulted in hyper-elevated T cell–mediated immunity, including lymphadenopathy, splenomegaly, and lymphocyte tissue infiltration (Wing et al., 2008). Many additional *in vivo* experiments demonstrated that Tregs with impaired CTLA-4 function failed to control the autoimmune responses in various autoimmune-prone experimental settings (Friedline et al., 2009; Ise et al., 2010; Jain et al., 2010; Schmidt et al., 2009; Walker, 2013).

CTLA-4 may exert its function on Tregs by modifying APC behavior. *In vitro* imaging studies identified Tregs aggregated around APCs (Onishi et al., 2008; Tang & Krummel, 2006). The persistent Treg–APC contacts in lymph nodes *in situ* are important for suppressing the T cell immunity, as demonstrated by the significant attenuation of the ability of these APCs to activate Teffs. There were no stable direct contacts between Tregs and $CD4^+CD25^-$ T helper cells (T_H cells), suggesting APCs are central for Treg function (Tang et al., 2006). In a CTLA-4-dependent manner, Tregs could induce expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO), and direct downregulation of B7 ligands on APCs, which are shared by co-stimulatory receptor CD28, probably by uptake of the ligands through trogocytosis (Bour-Jordan & Bluestone, 2009; Cederbom et al., 2000; Fallarino et al., 2003; Sprent, 2005).

It is now well established that CTLA-4 plays a significant role in Treg-mediated suppression of immunity. However, it is important to note reports that CTLA-4-deficient Tregs still possess suppressive function both *in vivo* and *in vitro* in different experimental settings (Stumpf et al., 2013; Verhagen et al., 2009; Walker, 2013). Although CTLA-4 is required for the function of Tregs, alternative compensatory mechanisms may exist in some circumstances (Wing et al., 2011). For example, depletion of CTLA-4 may upregulate other suppressive signaling molecules on Tregs, thus compensating/substituting for CTLA-4 in Treg function (Paterson et al., 2015).

In contrast to the critical role of CD28 in Tregs development and survival, CTLA-4 does not appear to be crucial for the generation and survival of Tregs, as Treg-specific deficiency of CTLA-4 did not show any deficit in Tregs development, expansion, and survival in a CTLA-4 Treg-intrinsic manner in non-inflammatory conditions (Bour-Jordan & Bluestone, 2009). However, there are also reports showing CTLA-4-deficient mice or administration of antagonistic anti-CTLA-4 mAb result in an amplified population of $CD4^+CD25^+$ Tregs, suggesting CTLA-4 may serve as a negative feedback loop to limit the population size of Tregs, most likely in a Treg-extrinsic manner (Bour-Jordan & Bluestone, 2009; Paterson et al., 2015; Schmidt et al., 2009; Tang et al., 2008).

PD-1/PD-L1 axis

The co-inhibitory receptor PD-1 belongs to the IgSF, and possesses a single IgV ectodomain and an intracellular domain containing two signaling motifs (Ishida et al., 1992). PD-1 has two ligands, PD-L1 and PD-L2, which both have a sequential IgV and IgC domains, and a cytoplasmic tail (Riella et al., 2012). It was recently reported that PD-L1 also interacts with B7-1, resulting in an inhibitory signal (Butte et al., 2007). Chemical

crosslinking experiments revealed that the interface of PD-L1/B7-1 overlaps with the interfaces of CTLA-4/B7-1 and PD-1/PD-L1, indicating they can possibly compete with each other (Butte et al., 2007). PD-L2 also interacts with RGMb (repulsive guidance molecule b) to attenuate respiratory immunity (Xiao et al., 2014). PD-1 expression is induced after activation of T cells, B cells, natural killer (NK) cells, and other APCs, including monocytes and myeloid dendritic cells (DCs) (Keir et al., 2008). PD-L1 has a broad expression profile, which includes most hematopoietic cells and a wide range of non-hematopoietic cell constitutively expressing PD-L1 (Cederbom et al., 2000). PD-L2 has a more restricted expression profile, with only a group of APCs inducing to express PD-L2 (Cederbom et al., 2000). Both PD-1 and PD-L1 are highly expressed on $Foxp3^+$ Tregs (Francisco et al., 2010).

The PD-1/PD-L1 axis has been found to play a role in the generation of peripheral pTregs. PD-L1, but not PD-L2, was required for the TGF-β-dependent conversion of naive T cells to Tregs, as $PD\text{-}L1^{-/-}$ DCs but not $PD\text{-}L2^{-/-}$ DCs failed to convert naive T cell in an *in vitro* experimental setting (Wang et al., 2008). In addition, $PD\text{-}L1^{-/-}$ APCs and $PD\text{-}L1^{-/-}PD\text{-}L2^{-/-}$ APCs retained similar minimal ability to convert naive CD4 T cells to pTregs. Conversely, PD-L1-coated beads were able to induce pTregs *in vitro*, indicating PD-L1 is important for pTregs induction (Francisco et al., 2009). PD-L1 positive T cells or irradiated K562 myeloid tumor cells were able to convert T_H1 cells into Tregs *in vivo*, whereas inhibition of PD-1 expression on T_H1 or inhibition of PD-1 signaling by SHP1/2 inhibitor prevented conversion during PD-L1 challenge (Amarnath et al., 2011). Moreover, murine vascular endothelium could induce peripheral $CD4^+CD25^+$ Tregs in a PD-L1-dependent fashion (Krupnick et al., 2005). In the EAE mouse model, the increased frequency of Tregs can be abrogated by PD-1 deficiency (Wang et al., 2010). These results indicate that the PD-1/PD-L1 axis contributes to peripheral tolerance by inducing peripheral pTregs.

Interestingly, in an autoimmune-like graft-versus-host disease (GVHD) model, it was shown that the donor Tregs in the recipients were predominantly expanded from tTregs, with few originating from pTregs (Yi et al., 2011). In addition, B7-1 rather than PD-1 expressed by donor Tregs was demonstrated to augment the proliferation and survival of tTregs through ligation with PD-L1 expressed on host APCs (Yi et al., 2011). However, in HCV chronically infected patients, upregulation of PD-1 on Tregs was found to be associated with the relatively lower expansion of Tregs *in vivo*. Blockade of PD-1/PD-L1 or PD-L1/B7-1 by antagonistic antibodies improved the *in vitro* proliferation and function of Tregs derived from the livers of patients, indicating that PD-1/PD-L1-related processes temper Tregs' function in chronic HCV infection patients (Franceschini et al., 2009). In contrast, in a lymphocytic choriomeningitis virus (LCMV) chronic mouse model, upregulation of PD-1 on Tregs facilitated the expansion and increased the suppressive capacity of Tregs (Park et al., 2015). Direct contact of PD-1 on Tregs and PD-L1 on $CD8^+$ T cells was partially responsible for the observed T cell suppression (Park et al., 2015). Many questions remain to be addressed, including reconciliation of the debate on the role of PD-1/PD-L1 in the function of Tregs. Additional studies in different settings need to be conducted, ideally exploiting mouse models with specific conditional knockout of PD-1/PD-L1/B7-1 in Tregs.

GITR

The co-stimulatory receptor GITR is a member of TNFRSF, which engages with the TNFSF ligand GITRL. Both human and mouse GITR has three tandem CRDs followed by a stalk region, a trans-membrane domain, and a cytoplasmic domain. Human GITRL exhibits an atypical expanded homotrimeic assembly, whereas mouse GITRL possesses an even more unusual dimeric structure (Chattopadhyay et al., 2007, 2008). Notably, unlike most members of the TNF/TNFR families, the human and murine GITR and GITRL do not cross react due to these distinct structural properties. In humans, GITRL protein expression can be detected in non-lymphoid tissues, including vascular endothelial cells, but cannot be detected on different PBMC subsets (Tuyaerts et al., 2007). In contrast, mouse GITRL protein is constitutively expressed on APCs, including DCs, freshly isolated macrophages, and subsets of B cells (Shevach & Stephens, 2006). The GITR receptor is constitutively expressed at high levels on Tregs and at low levels on other $CD4^+$ and $CD8^+$ T cells. Upon TCR ligation, GITR is upregulated on $CD4^+$ and $CD8^+$ T cells, with peak expression occurring 24–72 hours after TCR activation (McHugh et al., 2002; Shevach & Stephens, 2006).

Administration of anti-GITR antibodies (polyclonal or agonistic monoclonal) in mice abrogated the immunological tolerance conferred by Tregs, demonstrating a functional role for GITR in regulating Treg-mediated tolerance (McHugh et al., 2002; Shimizu et al., 2002). In another study using mice with advanced tumors, a single treatment with agonist anti-GITR mAbs evoked effective tumor immunity, resulting in tumor eradication. Stimulation of GITR reduced the $Foxp3^+$ Tregs in tumors, hampered Treg-mediated suppression, and enhanced $CD4^+$ and $CD8^+$ effector T cells infiltration in tumors (Ko et al., 2005). Using combinations of wildtype and GITR-deficient mice, one study demonstrated that the increased resistance of Teffs to Tregs after GITR stimulation *in vitro* and *in vivo* was primarily due to GITR on Teffs rather than Tregs (Ephrem et al., 2013; Stephens et al., 2004). Indeed, stimulation of GITR by different kinds of agonist reagents augmented Teffs proliferation, cytokine production, and survival (Igarashi et al., 2008; Kanamaru et al., 2004). In addition, GITR-deficient Tregs were not compromised in their ability to inhibit T cell expansion *in vitro* (Ephrem et al., 2013; Stephens et al., 2004). Thus, GITR may primarily control the Treg-mediated suppression in a Treg-extrinsic and not a Treg-intrinsic manner.

Though GITR may not be essential for Treg function, GITR can promote the expansion of Tregs. $GITR^{-/-}$ mice harbored normal numbers of Tregs in the thymus compared with wildtype mice, but about 33% fewer $CD25^+CD4^+$ Tregs in the spleen and peripheral lymph nodes, indicating GITR may contribute to the homeostasis of peripheral Tregs (Ronchetti et al., 2004; Stephens et al., 2004). GITRL-Fc treatment resulted in a dramatic expansion of Tregs and a mild expansion of Tconvs in naive mice, though the increase of Tregs frequency was transient and the percentage of Tregs returned to normal after the treatment (Ephrem et al., 2013). In a B cell–specific GITRL transgenic mouse model, in which GITRL expression was driven by the CD19 promoter, both the numbers of $CD4^+$ Tregs and Teffs were increased due to increased proliferation (van Olffen et al., 2009). B cells could restore the numbers of Tregs in EAE mouse model through the expression of GITRL and maintain tolerance (Ray et al., 2012). Thus, GITR may play a role in self-tolerance by adjusting the relative populations of Tregs and Teffs.

OX40

OX40 is another co-stimulatory receptor belonging to the TNFRSF. The ectodomain of OX40 contains four tandem CRDs, which engage the TNF ligand OX40L. Similar to human GITRL, OX40L possesses an atypical expanded homotrimeric organization, which is able to interact with three OX40 through the grooves between each protomer (Compaan & Hymowitz, 2006). OX40L is predominantly expressed on APCs, including DCs, activated B cells, microglia, and vascular endothelial cells (Takeda et al., 2004; Watts, 2005). OX40 expression is induced in activated T cells, whereas it is constitutively expressed on Tregs (Piconese et al., 2010).

There is considerable evidence supporting the role of OX40 in impairing the suppressive function of Tregs. OX40L transgenic mice, which express OX40L on T cells, spontaneously developed IBD-like colitis, whereas blockade of OX40/OX40L interaction or transfer of $CD4^+CD25^+$ Tregs prevented the disease (Malmström et al., 2001; Murata et al., 2002; Read et al., 2000). Further studies demonstrated Teffs were more resistant to Tregs when exposed to OX40L cells or agonistic anti-OX40 mAbs (Takeda et al., 2004). In GVHD models, triggering of OX40 inhibited the suppressor function of Tregs, possibly by reducing Foxp3 gene expression (Valzasina et al., 2005; Vu et al., 2007). In the context of cancer immunotherapy, the use of agonist anti-OX40 mAbs alone or combined use of cyclophosphamide (CTX) and agonist anti-OX40 mAbs reduced the suppressive immunity imposed by Tregs in the tumor and elicited tumor clearance (Hirschhorn-Cymerman et al., 2009; Piconese et al., 2008). In addition, OX40 signaling was shown to inhibit the TGF-β and antigen-mediated conversion of naive CD4 T cell to Tregs (So & Croft, 2007).

In contrast, other studies identified OX40 as a critical factor in stimulating Treg proliferation. For example, in a colitis mouse model, OX40 was found preferentially expressed on intestinal T cells and promote the accumulation of $Foxp3^+$ Tregs in the colon and suppressed the colitis (Griseri et al., 2010). OX40 signaling also promoted the fitness of Tregs by optimizing the Tregs responsiveness to IL-2, resulting in the inhibition of lymphopenia-driven colitis (Piconese et al., 2010). Though the tumors were eradicated after combination therapy, expanded Tregs were observed in the periphery (Hirschhorn-Cymerman et al., 2009). Interestingly, it has been reported that in the presence of Th1/2 cytokines, OX40 stimulation could block TGF-β-mediated conversion of activated T cells to Tregs, whereas in the absence of IFN-γ and IL-4, OX40 signaling enhanced the accumulation of Tregs, suggesting the cytokine milieu may be a key consideration for reconciling these disparate results (Ruby et al., 2009).

Other co-stimulatory and co-inhibitory molecules

Accumulating evidence supports the view that most co-stimulatory and co-inhibitory molecules, including members from both IgSF and TNFSF/TNFRSF, impact on Treg function, either primarily or secondarily to their effects on Tconvs (Figure 1). The co-inhibitory molecule LAG-3 (lymphocyte activation gene-3), which is constitutively expressed on Tregs, plays a crucial role in suppressing Teffs, possibly by engaging the MHC-II expressed DCs and inhibiting DCs activation (Huang et al., 2004; Liang et al., 2008). LAG-3 defines a population of active $CD4^+CD25^+Foxp3^+$ Tregs, which is expanded at tumor sites in cancer patients (Camisaschi et al., 2010). Though the

receptor for co-inhibitory ligand B7x has not been published, knockout of B7x reduced the number of tumor-resident infiltrating Tregs (Abadi et al., 2013). Conversely, administration of B7x-Ig proteins promoted the function and expansion of Tregs in the central nervous system (CNS) in an EAE mouse model (Podojil et al., 2013). Co-inhibitory molecule Tim-3 (T cell immunoglobulin mucin domain-3) was found expressed in a subset of $Foxp3^+$ Tregs and the expression of Tim-3 was associated with higher Treg suppressor functions (Gupta et al., 2012; Sakuishi et al., 2013). Similarly, expression of co-inhibitory molecule TIGIT (T cell Ig and ITIM domain) on Tregs enhanced the suppressive phenotype of Tregs in tumor tissues (Kurtulus et al., 2015). The co-stimulatory receptor ICOS (inducible co-stimulatory molecule) promotes the expansion of Tregs through interaction with ICOS ligand, which further inhibits tumor immunity (Conrad et al., 2012; Faget et al., 2012; Martin-Orozco et al., 2010).

In addition to members of IgSF mentioned in the last paragraph, members of the TNFSF/TNFRSF also modulate Treg homeostasis and function. The co-stimulatory receptor CD27 interacts with its ligand CD70 to promote the expansion of Tregs through reducing the apoptosis of Tregs and inducing IL-2 production from Teffs, which further reduce the adaptive T cell response against tumors (Claus et al., 2012). The co-stimulatory receptor CD30 is critical for Treg-mediated suppression, as CD30-deficient Tregs exhibited significantly compromised effect in preventing GVHD (Dai et al., 2004; Zeiser et al., 2007). The co-stimulatory receptor CD40 is important for the expansion of Tregs, as stimulation of CD40 by soluble CD40L (CD40 ligand) significantly increased the expansion of Tregs *in vitro* and abrogation of CD40/CD40L interaction by blocking antibody or CD40 knockout impaired Treg expansion (Huang et al., 2012; Pan et al., 2010). Receptor 4-1BB is quickly upregulated in activated $CD4^+CD25^+$ Tregs and stimulation of 4-1BB by 4-1BBL (4-1BB ligand) can dramatically expand the Tregs *in vitro* (Schoenbrunn et al., 2012; Elpek et al., 2007). Treatment of mice with agonistic mAbs against co-stimulatory receptor DR3 (death receptor 3) leads to dramatic expansion of Tregs but not Teffs, suggesting a role of DR3 in stimulating Treg proliferation (Schreiber & Podack, 2013). HVEM (herpesvirus entry mediator) expressed on Tregs can interact with gD (glycoprotein D) of HSV-1 (herpes simplex virus-1) to promote Treg proliferation and activation. Additionally, HVEM is also able to interact with IgSF member BTLA to enhance the suppression of Teffs conferred by Tregs (Pasero et al., 2009; Sharma et al., 2014; Tao et al., 2008).

Concluding remarks

With the emerging promise of co-inhibitory molecule (immune checkpoint) inhibitors in cancer immunotherapy, this area has become a hotbed of activity, with enormous efforts focused on the discovery of new receptors and ligands, and the development of new strategies for targeting these molecules (Assal et al., 2015; Ohaegbulam et al., 2015). There is convincing evidence that some of these checkpoint inhibitors also have therapeutically relevant effects on Tregs. Anti-CTLA-4 mAbs with the IgG2a isotype showed enhanced ability compared with IgG2b and IgG1 versions in antitumor activity by mediating a rapid depletion of Tregs and concomitant activation of Teffs at tumor sites (Selby et al., 2013). Two other studies also indicated Fc-γ-dependent elimination of Tregs improved the

efficacy of anti-CTLA-4 mAbs–mediated antitumor immunity in mice (Bulliard et al., 2013; Simpson et al., 2013). Consistent with these reports, in cancer patients treated with Ipilimumab, the clinical benefits were correlated with the ratio of Teffs to Tregs (Hodi et al., 2008; Liakou et al., 2008).

Reducing the suppressive environment conferred by Tregs is an important strategy in cancer immunotherapy (Kim et al., 2014). GITR and OX40 are attractive targets for compromising Tregs, as they are both co-stimulatory receptors to augment the activity of Teffs, whereas at the same time they are expressed constitutively on Tregs to inhibit the suppressive function of Tregs in tumor environment. One group showed that by activation of GITR with agonist mAbs, Tregs became less effective at suppressing Teffs, which led to effective antitumor immunity (Ko et al., 2005; Shimizu et al., 2002). Agonist anti-OX40 mAbs has also been demonstrated to effectively impair suppressive function of Tregs (Hirschhorn-Cymerman et al., 2009; Piconese et al., 2008), and targeting GITR and OX40 in clinical trials.

Combination immunotherapies aim to target multiple co-stimulatory or co-inhibitory molecules to augment the antitumor activity of Teffs while reducing the immune suppressive features of the tumor microenvironment. With the realization of the distinct effects of some co-stimulatory and co-inhibitory molecules on Teffs and Tregs, more comprehensive mechanistic considerations must be taken into account when designing novel immunotherapeutic strategies to treat malignancies. Nevertheless, blocking co-inhibitory molecules CTLA-4, PD-1/PD-L1 axis, B7x, and other checkpoint molecules abrogates the co-inhibitory signals to Teffs, at the same time also suppresses the function of Tregs. Combination immunotherapy using the checkpoint inhibitors in combination with reagents selectively targeting Tregs can possibly improve treatment benefits. Indeed, selective depletion of Tregs by anti-CTLA-4 mAbs did augment the antitumor immunity (Bulliard et al., 2013; Selby et al., 2013; Simpson et al., 2013). In summary, understanding of the impact of co-stimulatory and co-inhibitory molecules on Tregs will assist the development of new strategies targeting Tregs to complement the combination cancer immunotherapies.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Clinical Cancer Research

Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy

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Abstract

B7-H3 (CD276) is an important immune checkpoint member of the B7 and CD28 families. Induced on antigen-presenting cells, B7-H3 plays an important role in the inhibition of T-cell function. Importantly, B7-H3 is highly overexpressed on a wide range of human solid cancers and often correlates with both negative prognosis and poor clinical outcome in patients. Challenges remain to identify the receptor(s) of B7-H3 and thus better elucidate the role of the B7-H3 pathway in immune responses and tumor evasion. With a preferential expression on tumor cells, B7-H3 is an attractive target for cancer immunotherapy. Based on the clinical success of inhibitory immune checkpoint blockade (CTLA-4, PD-1, and PD-L1), mAbs against B7-H3 appear to be a promising therapeutic strategy worthy of development. An unconventional mAb against B7-H3 with antibody-dependent cell-mediated cytotoxicity is currently being evaluated in a phase I clinical trial and has shown encouraging preliminary results. Additional therapeutic approaches in targeting B7-H3, such as blocking mAbs, bispecific mAbs, chimeric antigen receptor T cells, small-molecule inhibitors, and combination therapies, should be evaluated, as these technologies have already shown positive results in various cancer settings. A better understanding of the B7-H3 pathway in humans will surely help to further optimize associated cancer immunotherapies. *Clin Cancer Res; 22(14); 3425–31. ©2016 AACR.*

Background

During an immune response, naïve T cells engage their T-cell receptor (TCR) to interact with a complex of MHC and peptide expressed by antigen-presenting cells (APC). This first signal is not sufficient to trigger full T-cell activation. A second signal is provided by the interaction of costimulatory molecules (most importantly B7-1/2 and CD28), leading to full T-cell activation. Following activation, coinhibitory molecules, such as CTL-associated protein 4 (CTLA-4), function to restrain T-cell responses, resulting in T-cell exhaustion and tolerance. Interactions between members of the B7 ligand family and the CD28 receptor family provide T-cell costimulation and coinhibition, regulating T-cell activation and tolerance, exhaustion and effector function, differentiation, and memory generation. B7-H3, also known as CD276, is an immune checkpoint molecule belonging to the B7-CD28 pathways.

Structure and functional significance of the B7-H3 pathway

B7-H3 is a type I transmembrane protein encoded by chromosome 9 in mice and 15 in humans. The extracellular domain is composed of a single pair of immunoglobulin variable domain and immunoglobulin constant domain in mice (2IgB7-H3 isoform) and two identical pairs in human (4IgB7-H3 isoform) due to exon duplication (1, 2). The intracellular tail of B7-H3 is short and has no known signaling motif. B7-H3 was first described in humans (3) and then in mice (2) but is universally expressed among species (4). A soluble form, cleaved from the surface by a matrix metallopeptidase (MMP; ref. 5) or produced through alternative splicing of the intron (6), is also detectable in human sera.

B7-H3 is expressed on many tissues and cell types. At the mRNA level, it is ubiquitously found in such nonlymphoid and lymphoid organs as the liver, heart, prostate, spleen, and thymus. Despite broad mRNA expression, protein expression is limited at steady state, suggesting the presence of an important posttranscriptional control mechanism. B7-H3 is constitutively found on nonimmune resting fibroblasts, endothelial cells (EC), osteoblasts, and amniotic fluid stem cells. Moreover, B7-H3 expression is induced on immune cells, specifically APCs. In particular, coculture with regulatory T cells (7), IFNγ, lipopolysaccharide (LPS), or anti-CD40 *in vitro* stimulation (8) all induce the expression of B7-H3 on dendritic cells (DC). Monocytes and monocyte-derived DCs upregulate B7-H3 after LPS stimulation or cytokine-induced differentiation, respectively (9). In addition, B7-H3 is also detected on natural killer (NK) cells, B cells, and a minor population of T cells following PMA/ionomycin stimulation (1).

The B7-H3 pathway has a dual role in contributing to the regulation of innate immune responses. One study found that neuroblastoma cells express B7-H3 on their cell surface, which protect them from NK cell–mediated lysis (10). Another group argues that B7-H3 costimulates innate immunity by augmenting proinflammatory cytokines release from LPS-stimulated monocytes/macrophages, in both a Toll-like receptor 4- and 2–dependent manner (11). The role of B7-H3 in controlling the innate immunity is clearly complex and requires more elucidation.

A larger body of literature suggests that B7-H3 plays an important role in T cell–mediated adaptive immunity, although the nature of its signalling remains controversial (12). A

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costimulatory role of B7-H3 on human T cells was initially reported *in vitro* (3). Murine studies showing B7-H3 worsens experimental autoimmune encephalomyelitis (EAE), arthritis, bacterial meningitis, and chronic allograft rejection (13–15) supported this claim. However, subsequent studies have mostly shown that B7-H3 acts as a T-cell coinhibitor. B7-H3 inhibits polyclonal or allogeneic CD4 and CD8 T-cell activation, proliferation, and effector cytokine production (IFNγ and IL2) in mice and humans. This negative regulation of T cells is associated with diminished NFAT, NF-κB, and AP-1 transcriptional factor activity (16). Researchers from independent studies using either protein blockade or gene-knockout mice have reported that B7-H3 ameliorates graft-versus-host disease, prolongs cardiac allograft survival, reduces airway hypersensitivity, and delays EAE onset, especially by downregulating Th1 responses (8, 17, 18). These examples lend more credence to the coinhibitory nature of B7-H3.

The receptor(s) for B7-H3 has yet to be discovered (19, 20). Nevertheless, the crystal structure of mouse B7-H3 reveals that its receptor engagement on T cells involves the particular segment connecting F and G strands (the FG loop) of the immunoglobulin variable domain of B7-H3 (19). Moreover, B7-H3 crystallizes as a glycosylated monomer but also undergoes an unusual dimerization *in vitro*. Together, the nature of the receptor(s), differences in cellular context, and various disease models certainly account for the discrepancies in the function of the B7-H3 pathway in regulating both innate and adaptive immunity during homeostasis and inflammation.

Beyond the immune system, the B7-H3 pathway has a nonimmunologic role in promoting osteoblastic differentiation and bone mineralization in mice, ensuring normal bone formation (21). Indeed, B7-H3 knockout mice had reduced bone mineral density and were more susceptible to bone fractures compared with wild-type mice. Furthermore, similar to other immune checkpoints of the B7-CD28 pathways, B7-H3 is also expressed in human cancers and participates in tumorigenesis through modulation of both immune and non–immune-related pathways.

B7-H3 in the tumor microenvironment and immune evasion

Numerous studies have described B7-H3 overexpression in human malignancies, including melanoma (22), leukemia (23), breast cancer (24), prostate cancer (25), ovarian cancer (26), pancreatic cancer (27), colorectal cancer (28), and other cancers. As detected by immunochemistry technique, more than 60% and up to 93% of patient tumor tissues display aberrant expression of B7-H3 in the vast majority of cancer types (Table 1), while limited expression is seen on normal healthy tissues. Within positively stained samples, B7-H3 is found on the membrane, in the cytoplasm, or within the nucleus of cancer cells but also on the tumor-associated vasculature. In a study of more than 700 colorectal cancer patients, cytoplasmic/membrane and stromal expression were respectively seen in 86% and 77% of the samples, whereas nuclear expression of B7-H3 in cancer cells was present in 27% of the samples (29). In most studies, the intensity of the B7-H3 staining was further quantified and ranged from low to high expression. Finally, association studies investigated potential clinical correlation between tumor-associated B7-H3 and disease severity. Various clinicopathologic parameters were assessed, including tumor size, metastasis, cancer stage, survival, and recurrence rate. In most cases, a high expression of B7-H3 was correlated with bad prognosis and poor clinical outcome. One study with more than 800 prostate cancer patients revealed that patients with strong B7-H3 expression on tumor cells had a significantly increased risk of disease spread at the time of surgery, clinical cancer recurrence, and cancer-specific death (25). B7-H3 expression in lung cancer was associated with a lower number of tumor-infiltrating lymphocytes and with lymph node metastasis, suggesting a role for B7-H3 in immune evasion and tumorigenesis (30). Importantly, B7-H3 protein expression in tumors is known to be modulated by miR-29 (31), upregulated upon IFNγ stimulation (32), and potentially increased by immunoglobulin-like transcript 4 signaling (33).

To date, the molecular mechanisms by which B7-H3 participates in tumor growth and immune evasion still remain elusive and need further investigation. Interestingly, aberrant glycosylation of B7-H3 was described in oral cancer. Its glycans, more diverse and with higher fucosylation, seem to interact better with DC-SIGN [DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin] and Langerin (34), proteins expressed on the membrane of DCs, suggesting a possible engagement and tolerization of DCs. Moreover, the cross-talk between lung cancer cells and tumor-associated macrophages, partially through IL10, induces B7-H3 membrane expression and inhibits T-cell antitumor immunity in mice (35). Besides its role in modulating tumor immunity, B7-H3 also has a nonimmunologic function in regulating tumor aggressiveness. It was shown to modulate migration, invasion, and adhesion to fibronectin of various cancer cells (36) through the Jak2/Stat3/MMP-9 signaling pathway (37). In addition, overexpression of B7-H3 in colorectal and breast cancer cells augments resistance to apoptosis by activating the Jak2/STAT3/survivin signaling pathway. This, in turn, weakens tumor cell sensitivity to the chemotherapeutic drug paclitaxel (38, 39). Furthermore, B7-H3 was shown to modulate the metastasis-associated proteins MMP-2, TIMP-1, TIMP-2, STAT3, and IL8 in melanoma cells (40). In hepatoma cells, B7-H3 targeted the epithelial-to-mesenchymal transition via the Jak2/STAT3/Slug signaling pathway (41). Finally, a recent study showed that decreased expression of B7-H3 reduces the glycolytic capacity and sensitizes breast cancer cells to AKT/mTOR inhibitors, unveiling a previously unknown link between B7-H3 and metabolism (42). Together, these mechanisms promote aggression and invasion of the tumor.

Clinical–Translational Advances

The precise role of B7-H3 in regulating the function of tumor-infiltrating immune cells and its activity in cancer cells has yet to be fully elucidated. This absence is due in large part to the conflicting studies that have demonstrated B7-H3 to be either costimulatory or coinhibitory in several disease models. In addition, the receptor(s) that interact with B7-H3 have yet to be identified, magnifying the scrutiny. However, there is no doubt that aberrant expression of B7-H3 consists of a possible biomarker and a promising immune checkpoint target for multiple cancer immunotherapy approaches (Fig. 1), as anticipated almost 10 years ago (43). The scientific community is beginning to explore its therapeutic role in cancer in a variety of ways.

Blocking mAbs

The B7 ligand and CD28 receptor families have become attractive targets for cancer immunotherapy, with specific emphasis placed on the development of mAb blocking B7-CD28 pathways. Blocking mAbs against the immune checkpoints CTLA-4,

Table 1. B7-H3 aberrant expression in human cancers and association with clinical–pathologic characteristics

Cancer type	B7-H3–expressing tumor tissues	Clinical correlation	References
Hepatocellular carcinoma	93.8 %	Poorer survival, increased recurrence	(32)
Pancreatic cancer	93.7%	Lymph node metastasis, lower differentiation grade	(27)
Prostate cancer	- 93%	- Disease spread, increased risk of clinical cancer recurrence, and cancer-specific death	(25)
	- 100%	- Larger tumor volume, extraprostatic extension, higher Gleason score, seminal vesicle involvement, positive surgical margins, >4-fold increased risk of cancer progression after surgery	(62)
Osteosarcoma	91.8%	Shorter survival and recurrence time, lower CD8 TIL	(63)
Breast cancer	- 90.60%	- Lymph node metastasis, advanced disease, IL10 in tumor cells	(64)
	- 80.55%	- Negative relation with VEGF, microvascular density for CD34, and tumor size	(24)
Colorectal cancer	- Cytoplasmic/membrane 86% Stroma 77% Nuclear 27%	- Reduced recurrence-free survival in TNM stage I	(28)
	- Cytoplasm 62% Membrane 46% Nuclear 30%	- Reduced metastasis-free, disease-specific, and overall survival	(29)
Ovarian carcinoma	Cytoplasm/membrane 83% Tumor endothelium 44%	High-grade serous histologic subtype, increased recurrence, and reduced survival	(26)
Endometrial cancer	75.7%	TIL infiltration, shortened overall survival	(65)
Oral squamous cell carcinoma	74.75%	Larger tumor size, advanced clinical stage, low survival rate	(34)
Cervical cancer	72.22%	Tumor size, positive correlation with FoxP3, negative correlation with IL2	(66)
Non–small cell lung cancer	- 69.5%	- Lymph node metastasis, TNM stage	(67)
	- 37.1%	- Lower TILs, lymph node metastasis	(30)
Bladder cancer	58.6%	No association	(68)
Clear cell renal cell carcinoma	Cancer cells 19% Tumor vasculature 18%	Large tumor size, advanced TNM stage, high nuclear grade, coagulative tumor necrosis, and capsular invasion	(69)
Glioma	Not specified	Malignancy grade	(70)
Melanoma	Not specified	Stage of melanoma, melanoma-specific survival in stages III and IV	(22)

NOTE: Not all clinical studies were included in this table due to the space limitation.
Abbreviations: TIL, tumor-infiltrating lymphocyte; TNM, tumor node metastasis classification.

programmed cell death protein 1 (PD-1), and PD-1 ligand 1 (PD-L1) have shown significant clinical success in patients with a variety of cancers (44–46). This same logic and success can be extended to B7-H3 as well (Fig. 1A). Blocking mAbs are effective because they either partially or completely neutralize inhibitory ligand-to-receptor interactions, thus allowing effector functions. Despite the fact that the B7-H3 binding partner(s) remains unknown and that mAbs generated against B7-H3 are specific to the protein, the ability of these mAbs to neutralize B7-H3 interactions and the signaling pathway remains unknown. Thus, currently, no blocking mAb against B7-H3 is available. Until this receptor (or receptors) is found, additional strategies in screening antibodies for neutralization capacity need to be developed.

Targeting B7-H3 through antibody-dependent cell-mediated cytotoxicity and antibody–drug conjugate therapies

The difficulties that have been encountered in creating blocking mAbs against B7-H3 have led to the optimization of antibodies against B7-H3 for therapy through alternative means (Fig. 1B). Enoblituzumab (MGA271), a mAb reactive to cancer-associated B7-H3 showed enhanced antitumor function through potent antibody-dependent cell-mediated cytotoxicity (ADCC) against a broad range of tumor cell types. In mice, weekly doses of MGA271 in both renal and bladder carcinoma xenografts resulted in sustained tumor growth inhibition, effects that were Fc mediated (47). Currently, an ongoing phase I study of enoblituzumab is being conducted in patients with refractory B7-H3–expressing tumors or B7-H3–expressing vasculature (trial NCT01391143). Preliminary results of the dose-escalation study indicate that as a monotherapy, the Fc-enhanced mAb enoblituzumab shows antitumor activity in several tumor types and modulates T cells by increasing the T-cell repertoire clonality in the peripheral blood of patients following treatment (48). Although enoblituzumab is not a blocking mAb and its success largely depends on ADCC, the results are encouraging and open the door for more clinical trials targeting this protein by way of mAbs.

Alternatively, mAbs can be stably conjugated to a biologically active cytotoxic drug or compound that induces cell death. Once the mAb binds the cell-surface antigen, the complex is internalized, releasing the cytotoxic substance and killing cancer cells. 8H9 is a mAb specific to B7-H3 that showed clinical success as an antibody–drug conjugate (ADC) after it was radiolabeled to iodine-131 (^{131}I) and administrated to patients with metastatic central nervous system (CNS) neuroblastoma (49). 8H9 also distinguishes itself from other B7-H3–specific antibodies in that it binds to the FG loop of B7-H3, a region critical to its immunologic function (50). Recently, 8H9 was humanized and affinity matured and maintained its ability to kill B7-H3–positive neuroblastoma cells *in vitro*. Two-fold and 5-fold enhancements in

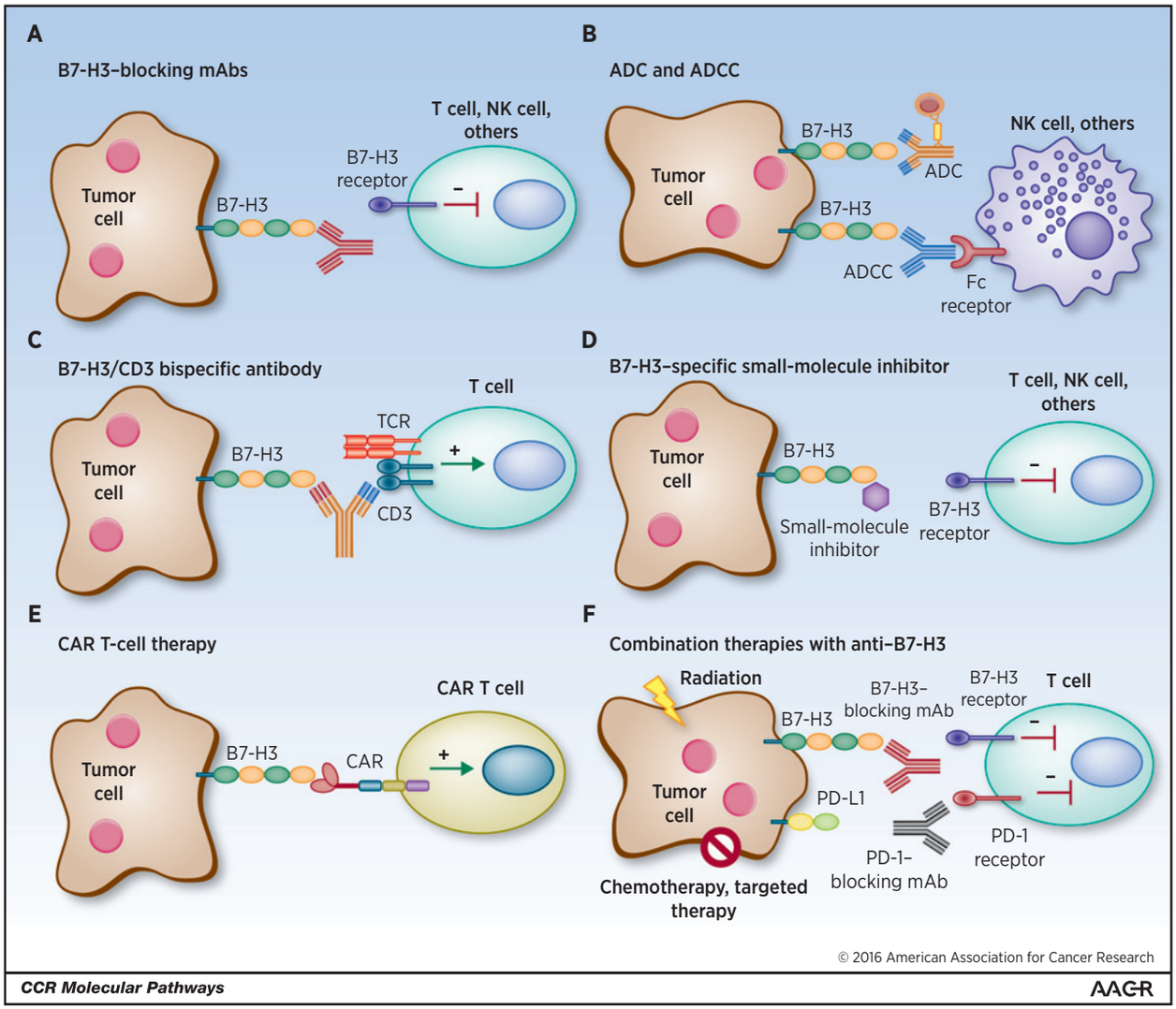

Figure 1.
Human cancer immunotherapy strategies targeting B7-H3 A, blockade of B7-H3 with blocking mAbs neutralizes inhibitory signaling in its unidentified receptor(s) in T cells, NK cells, and other immune cells enabling effector function. B, B7-H3–specific ADCC initiated by Fc receptor engagement of NK cells and other immune cells induces death of tumor cells. ADCs bind to B7-H3 expressed by tumor cells and are internalized and generate cytotoxicity to tumor cells. C, CD3/B7-H3–bispecific antibodies bind to tumor-expressed B7-H3 and crosslink the CD3 portion of the TCR complex, activating T cells in the tumor microenvironment for tumor cell death. D, small-molecule inhibitors may bind to specific regions of B7-H3, such as the FG loop of the IgV domain, inhibiting the ligand–receptor interaction between tumor cells and immune cells, thus blocking receptor signaling and restoring effector function of immune cells. E, engineered CAR T cells recognize membrane B7-H3 and directly kill tumor cells. F, blocking mAbs against B7-H3 in combination with radiation, chemotherapy, targeted therapy, or other immune checkpoint inhibitors synergize to generate more effective antitumor immune responses.

killing were observed in the affinity-matured and humanized 8H9 compared with the nonmatured and chimeric generations, respectively. Furthermore, the mAb was labeled with ^{131}I and injected into athymic nude mice xenografted with human neuroblastoma and showed successful biodistribution to the tumor (50). Currently, clinical trials with radiolabeled 8H9 are ongoing in patients with peritoneal cancers, gliomas, and advanced CNS cancers (NCT01099644, NCT01502917, and NCT00089245).

Bispecific antibodies

Bispecific antibodies are another suitable option beginning to pick up steam in the area of tumor immunotherapy. Bispecific antibodies are artificially generated antibodies composed of fragments of two distinct mAbs, thus combining two specificities. One arm can bind to the CD3 component of the TCR complex on T cells, while the other arm recognizes a tumor-specific antigen, for instance B7-H3, overexpressed on cancer cells (Fig. 1C). That way, T cells are recruited to the tumor site and activated to kill cancer cells (51). Given the upregulated expression of B7-H3 on multiple cancers, it seems like a promising option that should be pursued. Side effects of bispecific antibody treatment include an excessive inflammatory reaction due to cytokines produced by overactivated T cells but can be limited by corticosteroid administration.

Targeting B7-H3 with small-molecule inhibitors

With no current information known about the receptor(s) of B7-H3, the only viable target for disruption of this pathway is tumor-expressed B7-H3. In addition to conventional therapeutic mAbs, the roles of small-molecule inhibitors have also begun to gain interest in the immune-oncology field (52). Small-molecule inhibitors are low-molecular-weight organic compounds (dinucleotides, peptides, monosaccharides, etc.) that bind specific biological targets. They are readily used because of the advantages they offer in cheaper manufacturing costs, ease of delivery due to oral administration, greater tissue distribution due to size, and shorter half-life when compared with antibodies. Knowing that the receptor(s) of B7-H3 on activated T cells engages the FG loop of the IgV domain of B7-H3 (19), a small-molecule inhibitor could be designed to disturb this specific ligation area (Fig. 1D). Although often unpredictable, off-target effects can arise and should be assessed as thoroughly as possible to limit detrimental consequences.

Targeting B7-H3 with chimeric antigen receptor T cells

Another interesting way to target B7-H3 for immunotherapy is with chimeric antigen receptor (CAR) T-cell technology (Fig. 1E). This therapy recently had outstanding results in treating human refractive acute lymphoblastic leukemia (53, 54). Autologous T cells are engineered with a CAR targeting a tumor antigen and adoptively transferred to patients to kill cancer cells. So far, this technology has been successfully applied to hematologic cancers only. Although this area of research is challenging, efforts are being made to translate CAR T-cell therapy to the treatment of solid tumors. Importantly, the target must be highly overexpressed by the tumor and low or absent in normal peripheral tissues, as B7-H3, to avoid off-tumor effects. Engineered T cells would have to reach the tumor site and penetrate the stroma to specifically kill the targeted tumor cells. Moreover, CAR T cells would be exposed to the immunosuppressive tumor microenvironment, which could alter their function. Some optimizations of CAR T cells are currently being made and will hopefully help, either alone or in a combination therapy, to treat solid cancers (55). One clinical trial has evaluated the safety and antitumor activity of CAR T cells in patients with chemotherapy-refractory metastatic pancreatic cancer, with preliminary evidence of good tolerance and antitumor efficacy (56). Of note, a cytokine release syndrome has been described in some patients and must be addressed to fully ensure the safety of this technique.

Synergistic options with anti–B7-H3 therapy: Chemotherapy or targeted therapy, immune checkpoint inhibitors, and radiation

The clinical successes of mAbs blocking immune checkpoints, such as CTLA-4, PD-1, and PD-L1, have led to the rationale of combining these modalities with conventional therapeutics or additional checkpoint inhibitors, with the goal of synergizing their actions and improving patient survival. The most traditional therapeutic regimen for treating cancers has been with chemotherapy. Recent studies have shown that the combination of a variety of chemotherapeutics with checkpoint inhibitors displays great synergistic effects that enhanced the prospects of its full utilization in standard clinical practice. The combination of an anti–CTLA-4 mAb (ipilimumab) and the chemotherapeutic drug dacarbazine, when compared with dacarbazine plus placebo, led to improved overall survival in patients with metastatic melanoma (57). On the basis of a few preclinical animal studies, the combination of B7-H3 blockade and chemotherapy looks promising (Fig. 1F). Indeed, the silencing of B7-H3 through shRNA in an histiocytic lymphoma-derived human cell line, U937, in combination with the antineoplastic drug Ara-C, led to 80% tumor reduction compared with the 40% inhibition observed in wild-type U937 cells combined with Ara-C in a mouse xenograft model (58). Similarly, shRNA silencing of B7-H3 in a murine model of breast cancer, combined with the chemotherapeutic paclitaxel, led to an approximately 80% reduction in tumor growth compared with the untreated wild-type cells (38). In both studies, silencing B7-H3 significantly enhanced tumor cell chemosensitivity and drug-induced apoptosis. Moreover, exploiting the differences between normal cells and cancer cells through targeted therapy as opposed to conventional chemotherapy may also deliver exciting results as a combination strategy. Taken together, these studies provide a rationale for the potential synergistic effects between B7-H3 blockade and chemotherapy or targeted therapy for patients with a variety of cancers.

The combination of multiple immune checkpoint inhibitors as a means for treating cancers has also been emerging quite rapidly. A recent study has shown that the combination of anti–PD-1 mAb (nivolumab) and ipilimumab in patients with previously untreated melanoma resulted in significantly longer progression-free survival than ipilimumab alone (59). Furthermore, the combination of PD-1 and CTLA-4 blockade was able to demonstrate efficacy in patients with PD-L1–negative tumors compared with either agent alone. The expression pattern of B7-H3 contrasts greatly with that of the other checkpoint inhibitors in that the majority of B7-H3 can be found on tumor and tumor-associated tissue, while the others are expressed on immune cells, normal tissue, and cancerous cells. This difference in expression can be highly advantageous for generating not only local responses through the tumor-specific targeting of B7-H3, but also systemic activation of immune cells through additional checkpoint blockade, altogether potentially further enhancing antitumor immunity (Fig. 1F). Despite the fact that no studies are available yet in preclinical models, phase I clinical trials are under way to explore the safety of enoblituzumab in combination with either ipilimumab or anti–PD-1 (pembrolizumab) in patients with refractory cancer (NCT02381314 and NCT02475213).

Radiation is an additional avenue that can be looked at in combination with B7-H3 targeting in a future clinical setting (Fig. 1F). An anecdotal clinical report suggests that ipilimumab plus radiation cooperates to limit melanoma growth (60). Further studies confirmed these results in a small subset of melanoma patients treated with ipilimumab and radiation (61). Of note, resistance was commonly seen and explained by PD-L1 upregulation on the melanoma cells, causing T-cell exhaustion, and highlighting the need for a triple combination therapy. Another area for exploration is the potential synergistic effects of B7-H3 blockade and radiotherapy and its underlying mechanisms for future development of novel cancer immunotherapies (Fig. 1F).

Concluding Remarks

B7-H3 has both immunologic and nonimmunologic functions. Largely overexpressed in human tumor tissues, B7-H3

positively correlates with cancer severity and poor outcome. Compared with other immune checkpoints, the B7-H3 pathway not only regulates innate and adaptive immunity but also promotes cancer cell aggressiveness through various nonimmunologic functions. Therefore, B7-H3 seems to be a unique and interesting target for future cancer immunotherapies. One of the most promising therapeutic strategies may be the use of blocking mAbs against the B7-H3 pathway. Rather than administered alone, blocking mAbs are more likely to achieve synergistic antitumor effects if they are combined with a chemotherapeutic regimen or other checkpoint inhibitors. In parallel, finding its receptor(s) and better elucidating the involvement of the B7-H3 pathway in immune responses and cancer development is crucial, as this knowledge would help with the design of more effective therapeutic agents, with the ultimate goal of complete and durable treatment of human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E. Picarda, K.C. Ohaegbulam, X. Zang
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INVITED REVIEW

WILEY Immunological Reviews

The third group of the B7-CD28 immune checkpoint family: HHLA2, TMIGD2, B7x, and B7-H3

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Summary

The B7-CD28 family of ligands and receptors play important roles in T-cell co-stimulation and co-inhibition. Phylogenetically they can be divided into three groups. The recent discovery of the new molecules (B7-H3 [CD276], B7x [B7-H4/B7S1], and HHLA2 [B7H7/B7-H5]/TMIGD2 [IGPR-1/CD28H]) of the group III has expanded therapeutic possibilities for the treatment of human diseases. In this review, we describe the discovery, structure, and function of B7-H3, B7x, HHLA2, and TMIGD2 in immune regulation. We also discuss their roles in important pathological states such as cancers, autoimmune diseases, transplantation, and infection. Various immunotherapeutical approaches are emerging including antagonistic monoclonal antibodies and agonistic fusion proteins to inhibit or potentiate these molecules and pathways in cancers and autoimmune diseases.

KEYWORDS
B7-H3, B7x, HHLA2, immune checkpoint, immunotherapy, TMIGD2

1 | INTRODUCTION

In the past two decades, there has been a major advancement in understanding the functions of the immune system, and one of the foremost achievements is the development of new immune checkpoint inhibitors. Checkpoint molecules are now sufficiently numerous such that they can be divided into families. The B7-CD28 family can be phylogenetically divided into three groups[1,2]—Group I consisting of B7-1/B7-2/CD28/CTLA4 and B7h/ICOS; group II containing PD-L1/PD-L2/PD-1; group III including B7-H3 (CD276), B7x (B7-H4/B7S1), and HHLA2 (B7H7/B7-H5)/TMIGD2 (IGPR-1/CD28H). The B7-1/B7-2/CD28/CTLA-4 pathway is important in modulating central immune tolerance while PD-L1/PD-L2/PD-1, B7-H3, B7x, and HHLA2 are important in peripheral immune regulation. The discovery, understanding, and therapeutic manipulation of the CTLA4 and PD-1/PD-L1 pathways have led to important therapeutic advances in cancer immunotherapy in patients and have led to improved patient cures and survival.[3,4] In this review, we will focus on the advances of the new immune checkpoints in the third group of the B7 family namely B7-H3, B7x, HHLA2, and TMIGD2.

2 | B7-H3

B7 homolog 3 protein (B7-H3) also known as CD276 is an immune checkpoint molecule that belongs to the B7-CD28 family and was

*These authors contributed equally to this work.

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discovered in 2001.[5] This molecule has been associated with co-stimulatory as well as co-inhibitory functions in regulating T-cell responses. B7-H3 is encoded on chromosome 15 in humans[5] but in mice it is located on chromosome 9.[6] B7-H3 is universally expressed across various species from teleost fish to mammals, thus it is one of the most evolutionarily conserved B7 family members.[7]

It is a 316-amino acid type I transmembrane glycoprotein with a molecular weight of 45-66 kDa. The extracellular domain is composed of two identical pairs of the immunoglobulin constant (IgC) and variable (IgV) domain in humans due to exon duplication (four IgB7-H3) while it consists of a single pair in mice (two IgB7-H3). The intracytoplasmic tail is short and has no known signaling motif.[6,8,9] The B7-H3 receptor has not yet been identified.[10,11]

2.1 | Structure

Murine B7-H3 (mB7-H3) possesses an ectodomain composed of a single IgV domain and a consecutive IgC domain.[1,5] The structure of the ectodomain, and elucidation of the detailed biophysical properties of the molecule, provides the models for mapping the possible receptor-binding sites and functional analysis.[11]

The crystal structure of mB7-H3 construct, including both IgV and IgC domains, was determined to the resolution of 3 Å with the space group $P6_122$. The ectodomain of mB7-H3 was produced by *Drosophila* S2 cells and purified as a glycosylated monomer. However, mB7-H3 crystallized as an unusual dimeric form, resulting from the mutual swap of the IgV domain G strands between two adjacent molecules (Figure 1). The connection segment of the F and G strands adopts an extended "beta strand-like" conformation rather than the classical FG loop conformation of the IgV domains. Consequently, the crystal structure of the mB7-H3 IgV domain is formed by a "back sheet" (ABED strands) and a "front sheet" (C″C′CFG* strands), of which the G* strand is contributed by the neighboring mB7-H3 IgV domain. The mB7-H3 C-terminal IgC domain (residues 140-239) located on the C terminus of the ectodomain adopts the classical IgC folding with sheets ABED and CFG.[12]

Upon days of storage at high concentration at 4°C, monomeric mB7-H3 gradually forms a stable dimer in a concentration-dependent manner.[11] Consistent with the crystal structure of mB7-H3, of which the IgV FG loop residues adopt an unusual extended strand-like conformation, replacements f the residues by other residues in the FG loop perturb the dimerization, suggesting that the observed G loops exchanging in the crystal structure contributes to the dimerization of mB7-H3 in solution. Interestingly, both monomeric and dimeric mB7-H3 showed consistent abilities to inhibit T-cell proliferation with no significant difference. Thus, dimerization of mB7-H3 alone does not change the inhibitory function of the molecule.[11]

A canonical monomeric model of mB7-H3 was generated based on the crystal structure of the dimeric mB7-H3.[13] Two potential N-glycosylation sites (residues Asn91 and Asn104) are predicted on the IgV domain of mB7-H3 based on the sequence of the molecule. Significant electron density was observed near Asn91 and was identified as a single N-acetyl glucosamine (NAG). More electro-density was observed adjacent to residue Asn104 and was interpreted as two NAG and two mannose sugar residues. Both glycosylation residues and sugars are located in the "back sheet" of mB7H3 IgV domain (Figure 1). A chimera mB7-H3 mutant, of which the whole FG loop

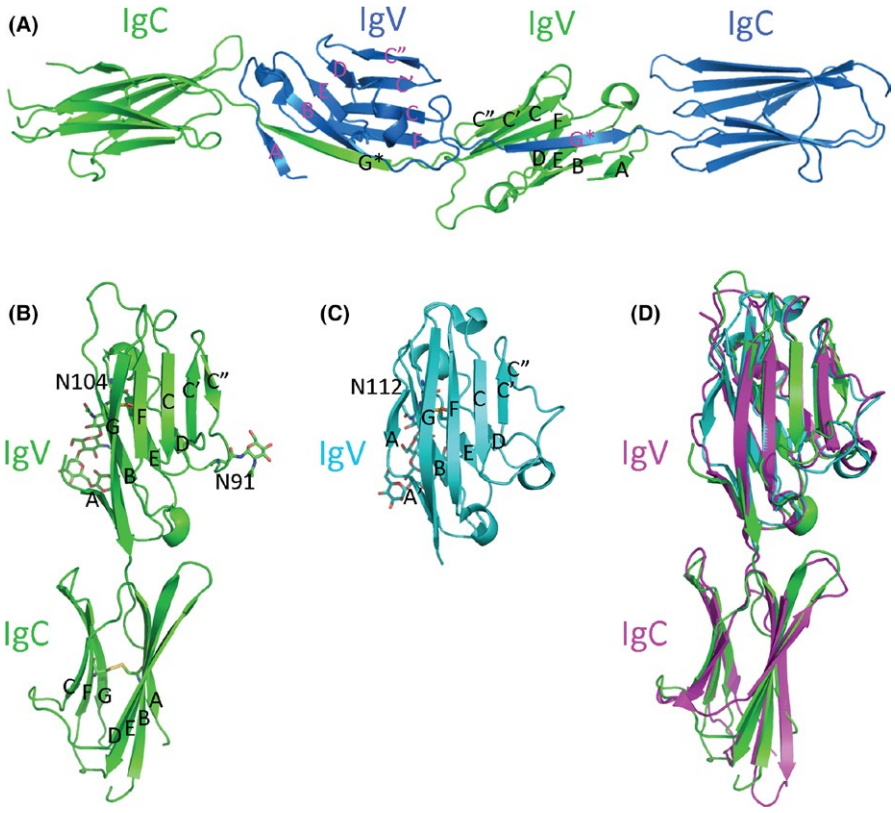

FIGURE 1 Structures of B7-H3, B7x, and PD-L1. A, Overall structure of the dimeric mB7H3 in the crystal (PDB entry 4I0K). The strands from each monomeric mB7H3 are colored and labeled differently. B, The generated model of monomeric mB7H3 based on the crystal structure from PDB entry 4I0K. C, The structure of the hB7x IgV domain (PDB entry 4GOS). D, Superimposition of the monomeric mB7H3, hB7x, and hPD-L1 (PD-L1 is from A chain of the PDB entry 3BIK). The disulfide bonds, carbohydrates, and the connecting Asn residues are shown as sticks

(residues 126-129; sequences: IQDF) was replaced to the cognate sequences from human PD-L1 (sequences: YGGA), completely lost the mB7-H3-mediated inhibitory activity. Surprisingly, alanine scanning targeted the residues on the "front sheet" of mB7-H3 IgV domain did not significantly change the inhibitory activity of mB7H3 as compared to the wildtype (WT) mB7H3.[11] These results indicate the individual mutation of the residues on the "front sheet" to alanine may not be sufficient to disrupt the receptor recognition, whereas replacement of the FG loop residues is significant enough to disrupt mB7-H3 function. This also demonstrates that the FG loop is important for mB7-H3-mediated inhibition.

2.2 | Expression

B7-H3 mRNA is widely expressed on many tissues such as those of the heart, thymus, prostate, testis, uterus, placenta, spleen, liver, pancreas, small intestine, and colon[14] (Table 1). Despite its broad mRNA expression, there appears to be a tightly regulated post-transcriptional control mechanism as protein expression is limited in steady state and maintained at low levels. The protein is constitutively expressed on non-immune resting fibroblasts, endothelial cells, osteoblasts, and amniotic fluid stem cells.[15] Its expression can be induced on immune cells such as T cells, natural killer (NK) cells, and antigen-presenting cells (APCs)

TABLE 1 Expression of B7-H3, B7x, and HHLA2 in tissues and cancers

Expression	B7-H3	B7x	HHLA2
Immune cells and tissues			
Dendritic cells	Strong[8,21]; induced [17,19]	Weak[1]; induced[71]	Negative[2]; induced[100]
Monocytes	Weak[21]	Induced[71]	Strong[2]
Macrophages	–	Weak[1]	Induced[100]
NK cells	Induced[8]	–	–
B cells	Negative[21]; induced [8]	Weak[1]; induced [71]	Induced[2]
T cells	Negative[21]; induced [8]	Weak[1]; induced [71]	Negative[2]
Osteoblasts	Strong[25]	Weak[110]	–
Tissues	Heart, thymus, prostate, testis, uterus, placenta, spleen, liver, pancreas, small intestine and colon[14]	Lung, testis, prostate, pancreas[1]	Placenta, colon, breast, small intestine, kidney, gallbladder[103]
Cancers			
Endothelial cells	Strong[111]	Strong[94]	–
Hepatocellular carcinoma	93.8%[33]	68.67% (HBV related)[112]	40%[103]
Pancreatic cancer	93.7%[39]	61.9%[39]	50%[103]
Colorectal cancer	C-86%, S-77%, N-27%[31] C-62%, N-30%[36]	48.21%[113]	37.5%[103]
Esophageal cancer	NS[114]	95.5%[115]	20%[103]
Renal cell carcinoma	19%, E-98% (clear cell)[45]	59.1%, E-81.5%[94]	33.33%[103]
Bladder cancer	58.6%[116]	49%[117]	40%[103]
Ovarian carcinoma	93%[37] E-44%[37]	100%[37] 85%[80]	50%[103]
Endometrial cancer	75.7%[34]	100% primary[118] 96% metastatic[118]	0%[103]
Cervical cancer	72.22%[48]	80.56%[48]	0%[103]
Breast cancer	90.60%[43] 80.55%[55]	95.4% primary, 97.6% metastatic[81]	56% TNBC[103] 70%[103]
Prostate cancer	93%[40] 100%[41]	99%[40]	33.33%[103]
Osteosarcoma	91.8%[35]	70.19%[110]	68%[108]
Oral squamous cell carcinoma	74.75%[38]	NS[119]	–
Lung cancer	69.5%[47] 37.1%[46]	43%[46] 31%[80]	66.67%[103] 66%[106]
Glioma	NS[49]	NS[82]	–
Melanoma	NS[44]	0%[80]	55.56%[103]
Thyroid	–	95.3%[120]	66.67%[103]

NS: not specified; %: % of tumor-expressing molecule; Weak/Strong: IHC staining of molecule.

including dendritic cells (DCs) and macrophages.[8,16,17] B7-H3 protein overexpression in tumor tissue was highly correlated with decreased expression of miR-29 as compared to normal tissues, and B7-H3 protein level could be modulated through manipulating miR-29 level in cultured cell lines, suggesting that a microRNA regulatory mechanism is involved in its differential expression.[18] In vitro, its expression, specifically on DCs, has been noted in coculture with regulatory T (Treg) cells,[19] interferon-gamma (IFN-γ), lipopolysaccharide (LPS), or anti-CD40 stimulation.[17,20] B7-H3 is upregulated on monocytes and monocyte-derived DCs after LPS stimulation or cytokine-induced differentiation, respectively[21] while it is detected on NK cells and B cells following ionomycin stimulation.[8,15] The limited expression of B7-H3 in normal tissues is consistent with the expression of other members of the B7 family.

2.3 | Function

Our appreciation of the roles of B7-H3 is evolving and the context of its expression is central to further defining the importance of this molecule. Its significance in innate and adaptive immunity is complex and still needs further elucidation.

2.3.1 | T cell

B7-H3 has been shown to have co-stimulatory and co-inhibitory activities in various studies. The initial study which led to the discovery of B7-H3 showed that B7-H3 increased proliferation of CD4 and CD8 T cells in the presence of anti-CD3 antibody. Moreover, B7-H3 played an important role in the generation of IFN-γ during activation of T cells.[5] In murine cancer models, B7-H3 has been shown to activate tumor-specific cytotoxic T lymphocytes (CTL) and hence potentiate antitumor immunity.[22] These studies showed that mB7-H3 can function as a co-stimulatory molecule.

Several studies show that B7-H3 predominantly causes T-cell co-inhibition. Similar T-cell proliferation assays showed that B7-H3 inhibited proliferation of CD4 and CD8 T cell in a dose-dependent manner and also reduced IL-2 and IFN-γ.[17] This inhibitory effect of B7-H3 was overcome by CD28-mediated co-stimulation. The expression of B7-H3 on APCs decreases T-cell proliferation. In mouse models of airway inflammation, B7-H3 downregulated Th1 but not Th2 responses, and B7-H3 gene knockout mice developed severe airway disease than the WT. In a mouse model of experimental autoimmune encephalitis (EAE), B7-H3-deficient mice or blocking B7-H3 through antibodies resulted in earlier onset and worse disease, respectively, through enhanced Th1 responses.[20] B7-H3, by modulating or inhibiting NFAT (nuclear factor of activated T cells), NF-κβ, and AP-1, can regulate T-cell receptor (TCR)-mediated gene transcription and hence cause T-cell co-inhibition.[22] These studies comprehensively show that B7-H3 causes T-cell co-inhibition. These effects of T-cell co-stimulation or co-inhibition by the same molecule could be due to multiple receptors, the presence of other co-stimulatory or co-inhibitory molecules, or the presence of different immune cells/cytokines leading to modulation of B7-H3 function.

2.3.2 | NK cell

B7-H3 has been shown to inhibit NK cell activity in tissue cultures and hence decreases NK cell function and therefore there should be a receptor on NK cells for B7-H3.[23] In glioma, higher grades are associated with a higher percentage of B7-H3 expression.[24] This study also showed that both soluble B7-H3 and cell-bound B7-H3 were able to inhibit NK cell-mediated lysis. In an in vivo model of B7-H3-silenced glioma cell lines, tumor formation was present but there was a marked decrease in the ability to form metastatic deposits when compared with control animals. This is important in tumor immunity, since when tumor cells lose HLA class I molecules, and hence escape cytolytic T-cell clearance, they become susceptible to NK cell-mediated lysis (Figure 2).

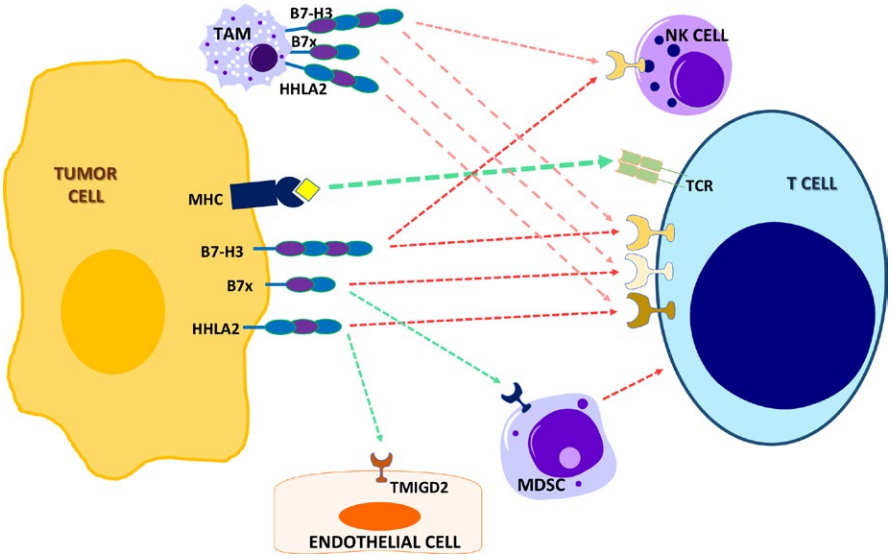

FIGURE 2 The third group of the B7-CD28 immune checkpoint family in the human cancer microenvironment. B7-H3 dampens (red broken line) NK cells and T cells, and may reprogram metabolism in cancer cells. B7x inhibits T cells and potentiates (green broken line) myeloid-derived suppressor cells (MDSC). HHLA2 restricts T cells and may promote angiogenesis through its receptor TMIGD2 on endothelial cells. Receptors for B7x and B7-H3 molecules have not yet been identified

2.3.3 | Other cells

B7-H3 also promotes osteoblast differentiation and bone mineralization. Despite having no gross skeletal abnormalities, the B7-H3 knockout mice displayed a lower bone mineral density in cortical bones compared with WT controls. These results show that B7-H3 is another factor in the growing interface between bone and the immune system.[25]

2.4 | B7-H3 in pathological states

2.4.1 | Infection

Soluble B7-H3 is elevated in patients with sepsis when compared with healthy persons and is even higher in patients who died from sepsis.[26] Whether soluble B7H3 represents a predictive or prognostic factor for sepsis is unclear as this was a small study and other confounding factors could have influenced the reported results. In patients with sepsis, serum B7-H3 levels correlate with increased levels of tumor necrosis factor α (TNF-α) and IL-6. TNF-α is the most potent inducer of B7-H3 release from monocytes. B7-H3 alone was unable to cause cytokine release from murine macrophages but it strongly augmented LPS-induced NF-κβ activation in a Toll-like receptor (TLR) 2- and TLR4-dependent manner.[26] Similarly, B7-H3 substantially augmented pro-inflammatory cytokine production in the development of pneumococcal meningitis in a murine model.[27] Thus, B7-H3 potentiates the inflammatory response in sepsis in murine models. Its levels are higher in humans during sepsis but its significance remains uncertain.

2.4.2 | Transplant

Allogeneic bone marrow transplant (BMT) is performed for hematological malignancies and achieves a cure; however, graft-vs-host disease (GVHD) after allogeneic transplant remains a major cause of death in transplant recipients. B7-H3 is upregulated in the colon, liver, and lung in mice, and in the intestine of GVHD patients. B7-H3 knockout recipients as well as recipients of B7-H3 knockout donor T cells had accelerated GVHD lethality. The increased GVHD lethality is a result of increased T-cell proliferation, colon inflammatory cytokines, and intestinal permeability. However, these T cells lacking B7-H3 are capable of providing graft-vs-leukemia (GVL) effects that are advantageous. Therefore, the B7-H3 pathway acts as a suppressor of acute GVHD. Approaches to increase B7-H3 expression soon after BMT to reduce GVHD and to decrease B7-H3 later post-BMT via the development of potent antagonistic B7-H3 monoclonal antibodies (mAbs) to enhance the donor lymphocyte infusion-mediated GVL effect may benefit transplant recipients.[28]

In solid organ transplants an early study showed B7-H3 had a co-stimulatory role in the alloimmune response. Its expression by cells promotes T cell-mediated immune responses and subsequently mediates the rejection of acute and chronic cardiac and islet cell allografts.[29] However, more recent results are consistent with an inhibitory function of B7-H3. Its signaling prolongs the survival of a fully MHC-mismatched cardiac model and promotes a shift toward a Th2 milieu.[30] Further studies are needed to clarify its role in solid organ transplants.

2.4.3 | Cancer

Aberrant expression of B7-H3 in various human malignancies including melanoma, glioma, leukemia, lung, pancreatic, renal, colorectal, ovarian, breast, gastric, and endometrial cancers was detected in 60-93% of the tumor tissue, while limited expression is seen on normal healthy tissues.[15] B7-H3 staining by immunohistochemistry (IHC) is present on the membrane, cytoplasm, nucleus, or vasculature of tumors. A study of more than 700 colorectal cancer patients showed cytoplasmic/membrane, stromal, and nuclear expression of B7-H3 in 86%, 77%, and 27% of the samples, respectively.[31] The molecular mechanisms that regulate its expression and specific functions remain unclear with both favorable and adverse outcomes associated with its expression in human tissues and mouse models.[16,32]

B7-H3 expression in hepatocellular carcinoma,[33] endometrial carcinoma,[34] osteosarcoma,[35] colon cancer,[36] ovarian carcinoma,[37] and oral squamous cell carcinoma[38] is associated with a shortened overall survival (OS). Its expression was also associated with disease progression or advanced stage in pancreatic cancer,[39] prostate cancer,[40–42] breast cancer,[43] melanoma,[44] clear cell renal cell carcinoma (RCC),[45] oral squamous cell carcinoma,[38] and non-small cell lung cancer (NSCLC).[46,47] Similarly, studies have also found a direct correlation with increased B7-H3 expression and tumor size, tumor grade, and rate of recurrence in humans.[31,48,49] Thus, there is ample evidence for the association of B7-H3 with poor prognosis in human cancers.

Although B7-H3-expressing cells are able to evade tumor immunity, the exact mechanisms are not yet known. Upregulation of B7-H3 is associated with impaired T-cell stimulatory function,[19,50] suppressed NK-mediated cell lysis,[24] increased IL-10 secretion,[43] decreased IL-12,[51] modulation of the Jak/Stat pathway[52] which contributes to immune suppression and evasion by tumors.

There are few early studies that associate B7-H3 expression with favorable clinical outcomes in cancer. In 102 patients with gastric cancer and 68 patients with pancreatic cancer, 58.8% and 88.2% expressed B7-H3, respectively. In both these studies, high levels of B7-H3 expression were associated with a significantly better prognosis.[53,54] Its expression was correlated with a higher number of tumor-infiltrating CD8 T cells in the pancreatic cancer patients, while in breast cancer B7-H3 suppressed tumor growth by inhibiting VEGF expression.[55] B7-H3 slowed tumor growth mediated by CD8 T cells and 50% had complete regression in mouse models of lymphoma and mastocytoma.[56,57] In HCC mouse models multiple distant tumor nodules regressed and tumor angiogenesis was inhibited.[58,59] In colon cancer survival time was prolonged, and tumor size as well as the occurrence of metastases was reduced.[60,61] Ablation of B7-H3 resulted in increased tumor burden in a spontaneous prostate cancer model.[62] These studies show that a high level of B7-H3 may be beneficial to anti-tumor immune responses.

There are several potential explanations for B7-H3's complex and conflicting immunomodulatory activity: (1) B7-H3 interacts with both inhibitory and stimulatory receptors; (2) it has different affinities for several receptors; (3) it has other aberrant isoforms or splice variants; and (4) genetic polymorphisms or differential glycosylation patterns may exist for the B7-H3 gene and protein.[16]

2.5 | Immunotherapy targeting B7-H3

Recent advances in immunotherapy targeting the B7-CD28 family members, CTLA-4/B7-1/B7-2 and PD-1/PD-L1 have been clinically successful and caused tumor regression.[63–65] Therapies targeting the B7-CD28 pathway are related to blocking the co-inhibitory signal between immune cells-immune cells and/or immune cells-tumor cells as well as the potentiation of the co-stimulatory signals. Progress in developing immunotherapies targeted at B7-H3 is deterred by the conflicting evidence of the role of this molecule in tumorigenesis. In addition, until the receptor is identified, therapy can only be targeted toward the molecule itself. Clinical trials with B7-H3-targeted immunotherapy are underway showing some promise. There are multiple modalities by which this molecule can be targeted as described below.[15]

Blocking mAbs such as nivolumab and ipilimumab have been effective and are now widely used clinically. Similar antibodies to B7-H3 are currently unavailable clinically and the ability of these antibodies to neutralize the signaling pathway remains unknown. Until the exact role of B7-H3 in specific tumors is defined, the development of functional blocking antibodies to B7-H3 will be a challenge.

Enoblituzumab (MGA271), a mAb against B7-H3 with potent anti-tumor activity through antibody-dependent cell-mediated cytotoxicity (ADCC), is being explored. This antibody showed tumor growth inhibition in B7-H3 expressing renal and bladder carcinoma xenografts.[66] There is currently an ongoing phase I trial being conducted in patients with refractory B7-H3 expressing tumors or vasculature (trial NCT01391143). The preliminary results of the dose escalation study show that it is well tolerated and has anti-tumor activity by increasing the T cell repertoire clonality in patients (http://www.macrogenics.com/enoblituzumab-anti-B7-H3/).

Similarly, an antibody drug conjugate with 8H9, a mAb radiolabeled to iodine-131 showed clinical success as salvage therapy in patients with metastatic central nervous system tumor called neuroblastoma. The patients received compartmental intrathecal antibody-based radioimmunotherapy and 83.3% of patients had prolonged survival.[67] Currently, Phase I clinical trials with radiolabeled 8H9 are ongoing in patients with peritoneal cancers, gliomas, and advanced CNS cancers (NCT01099644, NCT01502917, and NCT00089245). Recently a humanized affinity-matured potent form of this antibody (hu8H9) has been developed that binds to the FG loop of B7-H3, a region critical to its immunologic function.[68]

Synergistic therapy with a B7-H3 mAb and chemotherapy has met with preclinical success. In a murine pancreatic cancer model, B7-H3 blockade with the mAb MJ18 along with gemcitabine showed a synergistic anti-tumor effect without overt toxicity.[69] Two other preclinical animal studies have shown that silencing B7-H3 as well as utilizing conventional chemotherapy with cytarabine or paclitaxel led to 80% tumor reduction in murine models of histiocytic lymphoma-derived human cell line U937 and human breast cancer line, respectively, compared to WT models.[52,70] In both studies, silencing B7-H3 significantly enhanced tumor cell chemosensititvity and drug-induced apoptosis. Hence, this provides a rationale for the potential synergistic effects between B7-H3 blockade and chemotherapy for treatment of cancers.

The majority of B7-H3 is expressed on tumor cells and vasculature while the other checkpoint inhibitors are expressed on immune cells, normal cells, and tumor cells. Such phase I clinical trials are under way to explore the safety of enoblituzumab in combination with either anti-CTLA4 (ipilimumab) or anti-PD-1 (pembrolizumab) in patients with refractory cancer (NCT02381314 and NCT02475213).[15] This difference can be highly advantageous for combination therapy that can generate local responses through tumor-specific B7-H3 targeting, with additional checkpoint blockade-enhancing antitumor immunity.

2.6 | Summary of B7-H3

B7-H3 is predominantly a T-cell co-inhibitory molecule with a partial co-stimulatory function reported in some studies. It is a type I transmembrane protein with four extracellular domains due to exon duplication in humans. B7-H3 potently inhibits T cell and NK cell functions and also has a role in bone development. The receptor for B7-H3 has not yet been characterized but is presumed to be on T and NK cells. In human cancers, consistent with the function of B7-H3, most studies show that its expression is associated with poor prognosis while a few studies show that its expression is associated with a better prognosis. Currently antibodies targeting B7-H3 through an ADCC mechanism and B7-H3 antibody drug conjugates are in Phase 1 clinical trials in humans and the initial results are encouraging.

3 | B7x

B7x (B7-H4, B7-S1) belongs to the Immunoglobulin superfamily and the B7 family of ligands. It was discovered by us and others using bioinformatic analyses in search for proteins with homology to other members of the B7 family.[1,71,72] The human B7x (hB7x) consists of 282 amino acids and shares an 87% amino acid identity with mouse B7x which shows that this molecule is conserved from an evolutionary standpoint. It shares varying degrees of identity with human—B7-1 (12%), B7-2 (13%), B7 h (16%), PD-L1 (18%), PD-L2 (18%), and B7-H3 (24%).[1] Human B7x is located on chromosome 1p12/13.1. This is a type 1 transmembrane protein with a signal peptide in the N terminus, an extracellular domain with IgV- and IgC-like domains with four conserved cysteine residues and seven sites for N-linked glycosylation.

3.1 | Structure of B7x

The crystal structure of the hB7x IgV domain was determined to the resolution of 1.59 Å with the space group $P4_32_12$. The hB7x IgV

domain was expressed by *Drosophila* S2 cells and the purified protein crystallized as a monomeric form with each asymmetric unit containing one hB7x molecule.[73] The structure of hB7x IgV adopts a typical IgV organization,[12] which is characterized by "β sandwich" folding formed by a "back sheet" (ABED strands) and a "front sheet" (C"C'CFGA' strands) stabilized by a disulfide bond between B and F strand (formed by Cys56 and Cys130). A significant electron density, which could be fitted by five sugar residues of a branched glycan, is well defined near Asn112, corresponding to the glycosylation site at Asn112 as predicted by hB7x sequences. Notably, the large observed branched glycan covers a significant area of the "back sheet" surface (Figure 1). Similarly, a glycosylation site at the homologue position (Asn104 of B7H3) has been observed for the mB7H3 ectodomain.[11]

Crystal structure of hB7x provides a model for epitope mapping, which may further improve the efficacy of antibodies targeting hB7x. For example, 1H3 is an IgG1 mAb against B7x, which has been proved to inhibit tumor nodule formation and prolong the survival of mice inoculated with hB7x-expressing CT26 tumors.[74] The Fab of 1H3 is effective in blocking the B7x-mediated T-cell co-inhibition, suggesting 1H3 is a functional neutralizing antibody. Mutagenesis study showed that BC loop (residues Ile62 and Lys63) of the "back sheet" and FG loop (residue Ser135) of the "front sheet" define the minimal footprint for hB7x:1H3 interaction interface.[73] In combination of these data, it is very likely that the B7x receptor binds to the B7x IgV domain, which overlaps with the 1H3-binding interface.

Superimposition of the monomeric model of mB7H3 with human PD-L1 structure (from PDB entry 3BIK chain A) shows a similar organization with an overall Cα RMSD (root-mean-square deviation) ~2.7 Å (Figure 1). In addition, superimposition of hB7x IgV domain with human PD-L1 IgV domain resulted in even smaller Cα RMSD ~1.3 Å, with the most apparent differences in the loop regions (Figure 1). These indicate that overall structures of B7H3 and B7x resemble the other well-identified B7 family members. Most of the characterized B7 family members apply the "front sheet" of IgV domains to engage the receptors. For example, B7-1, B7-2, PD-L1, and PD-L2 all bind to the corresponding receptors through the "front sheet" of IgV domains.[75–79] Considering the obvious carbohydrates modifications of both B7-H3 and B7x on the "back sheet" of the IgV domains as identified in the crystal structures, it is very likely that B7-H3 and B7x may interact with their receptors through the "front sheet" of IgV domains, although more concrete evidence is required to support this hypothesis.

3.2 | Expression

B7x mRNA in immune cells is expressed by professional APC, bone marrow-derived DCs, peritoneal macrophages, splenic CD11c+ DCs, and splenic B cells.[1,80] B7x mRNA can be detected by PCR in most tissues but it is highly expressed in non-lymphoid organs like the lung, testis, and pancreas[1] (Table 1). Despite the high level of mRNA expression in most tissues, there is no detectable B7x protein in most healthy tissues.[80] Low levels of B7x expression were found in genital tract, lung, pancreas, and kidney, but were absent in other normal somatic tissues.[81]

The functional mechanisms of upregulation of B7x in glioma cells were explored.[82] IL-6 potently upregulated B7x expression in glioma cell lines. Stimulation of IL-6-mediated phosphorylation of STAT3 upregulated B7-H4 promoter activity, which was abrogated by knockdown of STAT3 with shRNA. This shows that STAT3 upregulates B7x transcription via its promoter and represents one mechanism of B7x regulation.[82]

3.3 | Function

3.3.1 | T cells

B7x negatively regulates T-cell responses. B7x decreases T-cell proliferation, which is a hallmark of T-cell activation, in a dose-dependent fashion by decreasing IL-2[72] and by inducing cell cycle arrest in T cells (a non-IL-2-dependent mechanism).[80] This is similar to CTLA-4 which can also inhibit TCR-induced T-cell proliferation by cell cycle arrest.[83] JunB which regulates IL-2 gene transcription after T-cell activation is decreased after B7x stimulation and this could be one mechanism of decreasing IL-2 production by B7x.[72] B7x inhibits T-cell growth, cytokine secretion, and cytolytic activity against allogeneic antigens in vivo.[80] The inhibitory effects of B7x could only be partially reversed by co-stimulation through the CD28 signaling pathway. B7x blocks the differentiation of naïve CD4 T cells to effector Th1 or Th17 cells by blocking IFN-γ and IL-17 production[84] (Figure 2). In all these studies it has been shown that B7x interacts with a receptor on activated T cells which is distinct form CD28, CTLA-4, ICOS, and PD-1.[1]

3.3.2 | Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are immature cells of the myeloid origin, which are mainly present in tumor and have a potent ability to suppress T-cell proliferation and cytokine production.[85] MDSC are divided into granulocytic (g-MDSC) and monocytic MDSC. In a B7x knockout mouse model, g-MDSC was the predominant population to infiltrate a tumor.[86] This g-MDSC makes up 60% of the tumor immune cells in WT mice while their composition is only 20% in B7x knockout mice, suggesting that the presence of B7x promotes expansion of MDSC. Moreover, B7x binds to g-MDSC indicating that MDSC has a receptor for B7x, there may be a distinct receptor on the MDSC different from the T cells.

3.4 | B7x in pathological states

3.4.1 | Autoimmune diseases and infection

B7x has been shown to dampen T-cell responses. To demonstrate the role of B7x in infections, the effects of Streptococcus pneumonia infection in B7x knockout mice were studied.[87] In the B7x knockout mouse model, the severity of *S. pneumonia* infection was markedly reduced when compared to WT mice. Control of infection in B7x knockout mice was associated with a marked increase in activated CD4 and CD8 T cells and fewer neutrophils in lungs, whereas

the susceptible WT mice had higher neutrophils with decreased CD4 and CD8 T cells. This suggests that B7x plays an important role in the dampening of immune responses to infection.

The role of B7x in the induction and maintenance of autoimmune diseases was studied in an EAE model. In vivo blockage of B7x with B7x antibodies during the T-cell priming phase led to an increase in the severity of the disease.[20] Similarly, EAE was severe in B7x-deficient mice when compared to WT mice due to the expansion of Th1 and Th17 cells during EAE induction without changing the Treg cell population leading to severe disease[88]

CD8 T cell-induced diabetes in B7x-deficient and B7x-overexpressing mouse models was used to study the roles of B7x in peripheral tissues. Mice lacking B7x developed a severe form of diabetes and transfer of antigen-specific CD8 T cells to B7x-overexpressing mice did not lead to diabetes. These studies showed that the CD8 T cells migrated to the pancreas of the B7x-overexpressing mice, but their proliferation, and thereby, tissue destruction is markedly reduced in the presence of B7x.[89]

The effects of B7x in an autoimmune kidney disease mouse model showed that B7x is expressed by several different kidney cells including tubular cells, podocytes, and glomerular epithelial cells at a low level. This expression is rapidly induced in the tubular cells after stimulation with LPS.[90] B7x knockout mice have an enhanced humoral immune response and develop severe renal injury after passive administration of antibodies against glomerular antigens. Correspondingly the macrophages in the spleen of B7x knockout mice were polarized to an inflammatory M1 phenotype. In accord with these studies, high levels of serum B7x were found in the sera of patients with rheumatoid arthritis and correlated with the disease severity score.[91] All these studies show that B7x regulates the induction and severity of autoimmune diseases and plays an important role in peripheral tolerance.

3.4.2 | Cancer

Aberrant expression of B7x has been detected in many human cancers which include—breast, lung, ovary, uterus, kidney, and others.[92] B7x is rapidly lost in in vitro cultures and hence most human[93] and mouse tumor cell lines are B7x negative.[73] Several human studies have studied the expression of B7x in cancers and its prognostic role in these malignancies. A few studies are described in detail below.

3.4.2.1 | Prostate cancer

Expression of B7x and correlation with clinical features were analyzed in a large study of 948 patients with localized prostate cancer treated with a pelvic lymph node dissection and radical retropubic prostatectomy.[40] B7x was highly expressed in the majority of prostate cancer with a strong intensity in 15% of cancer specimens. Strong intensity of staining for B7x in tumors was significantly associated with extracapsular extension, seminal vesicle invasion, and metastatic disease. These patients were also more likely to develop a biochemical recurrence (HR 1.38; 95% CI 0.94-2.02; *P*=.10), clinical recurrence (HR 2.22; 95% CI 1.27-3.87; *P*=.005) and had a higher probability of death from prostate cancer (HR 2.71; 95% CI 1.04-7.02; *P*=.04). Hence, B7x is highly expressed in prostate cancer and its strong expression in the tumor is associated with poor prognostic factors.

3.4.2.2 | Breast cancer

Similar to prostate cancer, B7x expression was detected in 95%-97% of primary and metastatic breast cancers. The staining intensity was much higher in invasive ductal carcinoma than in normal breast epithelium.[81] Even though B7x expression was associated with negative progesterone receptor status and history of neoadjuvant chemotherapy these factors are not necessarily predictive of a poor outcome in breast cancer.

3.4.2.3 | Renal cancer

In 259 RCC patients treated with nephrectomy, 59% tumor specimens exhibited B7-H4 staining.[94] Tumor cell B7-H4 expression was associated with adverse clinical and pathologic features, including constitutional symptoms, tumor necrosis, and advanced tumor size, stage, and grade. Patients with tumors expressing B7-H4 were three times more likely to die from their RCC (risk ratio=3.05; 95% confidence interval=1.51-6.14; *P*=.002). Interestingly, 81.5% specimens exhibited tumor vasculature endothelial B7-H4 expression, whereas only 6.5% of normal adjacent renal tissue vessels exhibited endothelial B7-H4 staining. Similarly, serum B7x was higher in 53 RCC patients (14.4 ng/mL) when compared to 18 control patients (2.7 ng/mL).[95] Median levels were significantly higher for patients with a tumor thrombus, positive lymph nodes, and distant metastases at nephrectomy. The median B7x levels also were progressively higher with increasing tumor-node-metastasis stage. These findings suggest that B7x is highly expressed in RCC, is associated with poor prognostic features, and since it is expressed in the vascular endothelium, whether this is involved in angiogenesis or metastases needs to be determined.

3.4.2.4 | Glioma

In glioma, a CNS tumor, higher grades of tumor are associated with higher B7x mRNA and protein expression when compared with normal brain tissue.[82] Similarly, increasing levels of soluble B7x was also found in CSF with increasing grades of glioma. CD133+ glioma-initiating cells mediated the expression of B7x on macrophages through IL-6 and IL-10 secretion. This results in a polarization of the macrophages toward a tumor promoting or a M2 phenotype, inhibition of T-cell proliferation and cytokine production, and led to reduced cytotoxicity of T cells. In a xenograft model of NOD/SCID mice, suppression of B7x leads to T-cell activation and tumor regression in mice. These experiments prove that B7x is an important checkpoint molecule in the development of glioma.

Based on these studies, B7x is expressed in most cancers and its expression is associated with a poor prognosis. Targeting B7x in human cancers is an attractive option for cancer immunotherapy.

3.5 | Immunotherapy targeting B7x

3.5.1 | Cancer

Anti-B7x therapy has been tested in mouse models in the form of anti-B7x single-chain fragment variables (scFv), ADCC and functional antibodies, antibody drug conjugates, and chimeric antigen receptor (CAR) T cell-B7x therapy. An anti-B7x antibody was generated to study the effects of inhibiting B7x in mouse cancer models. This antibody functions both as a blocking antibody to inhibit function and kill through an ADCC mechanism, and since B7x is evolutionally conserved, the antibody also inhibits hB7x. A colon carcinoma CT26 cell line which stably expressed B7x and induced experimental lung metastasis was tested with anti-B7x treatment.[73] The average number of metastasis was higher along with a poor survival in the B7x/CT26-injected mice than in the CT26 group. Treatment with Anti-B7x antibody (1H3 clone) significantly lowered pulmonary metastases and increased survival in the B7x/CT26 model. The anti-B7x antibody-treated mice had increased infiltration of antigen-specific cytotoxic T cell and NK cells in the lungs and decreased CD4 or CD8 T cells of the exhausted phenotype. Similarly, the proportion of MDSC infiltrating the tumor and cytokines including VEGF and TGF-β was also reduced. As expected, the B7x antibodies had minimal off target effects.

Single-chain fragment variables are recombinant antibodies with single antigen-binding domains and have the advantages of small size and versatility for in vivo targeting and binding to conjugates. Anti-B7x scFv fragments decreased the growth of ovarian tumors. These antibodies also stimulate tumor antigen-specific T-cell activation when tumor immune responses exist prior to therapy.[93]

A CAR T cell-B7x therapy was tested in a murine ovarian cancer model. It was not only very effective against human ovarian tumor xenografts but also had delayed lethal toxicity.[96] Post mortem analysis showed that the normal tissues showed increased expression of B7x protein. This late toxicity may be overcome by means of a switch receptor/chimera or by using suicidal gene therapy before the delayed onset of normal organ toxicity which is distinct from the anti-tumor effects. These therapies represent different approaches to target B7x in the tumor microenvironment.

3.5.2 | Autoimmune disease

In contrast to cancers where inhibition of B7x is beneficial, potentiation of B7x-induced immune suppression has been shown to suppress autoimmune diseases. A non-obese diabetic (NOD) mouse model was used to study the effects of B7x in development of type 1 diabetes.[97] NOD mice were injected with B7x immunoglobulin fusion protein (B7x-Ig) at 3 weeks, before the onset of diabetes. Early treatment of NOD mice with B7x-Ig resulted in a later development of diabetes. It did not prevent the onset of inflammation but rather decreased the amount of islet cell destruction by modulating the immune infiltrate. This is due to a decreased Th1 response mediated by upregulation of T regulatory cells in the pancreas while this did not happen systemically in the blood. In addition, B7x-Ig-treated mice had decreased numbers of Th17 cells and hence decreased levels of Th17-associated cytokine and an increase in IFN-γ which potently inhibits Th17 cells.[98] The treatment downregulates the Th17 pathway which is important in the development of autoimmune diseases.[99]

A similar approach was used to demonstrate that B7x-Ig effectively ameliorated EAE in mice by decreasing CD4 T cells and by increasing the number and function of T regulatory cells.[84] Specifically it blocked IFN-γ and IL-17 production and hence blocks differentiation of naive CD4 T cells to either a Th1 or Th17 phenotype. This could be used as therapeutic model to potentiate the B7x pathway for human demyelinating disorders like multiple sclerosis.

As previously described, a mouse nephritis model was used to demonstrate that B7x decreases autoimmune kidney injury.[90] Following nephrotoxic kidney injury, B7x-Ig inhibits the expansion of CD4 and CD8 T cells and restricts macrophage polarization to a M1 phenotype. The M1 phenotype is likely to cause autoimmune diseases in experimental models. B7x-Ig treatment also decreases the levels of various cytokines involved in autoimmune nephritis namely—CXCL13, IRF5, ICOS-L, and TGF-β. All these studies show that therapeutic potentiation of B7x pathway in humans with preexisting autoimmune diseases or in those with a high risk of developing autoimmune disease is likely to be beneficial.

3.5.3 | Summary of B7x

B7x is a T cell co-inhibitory molecule with a 15%-25% homology to other members of the B7 family. B7x is a type 1 transmembrane protein with two extracellular domains. B7x inhibits T cell function through IL-2-dependent and -independent mechanisms and inhibits proliferation of T cells by causing cell cycle arrest. One mechanism of induction of B7x is through IL-6. IL-6 phosphorylates STAT3 which in turn binds to the B7x promoter and upregulates B7x. The receptor for B7x has yet to be unidentified but is distinct from the other members of the B7 family. Even though B7x mRNA is expressed in multiple tissues, B7x protein is rarely detected in normal tissues. Multiple in vivo studies have shown that B7x is important for induction and maintenance of peripheral tolerance in tissues and hence plays an important role in autoimmunity. Stimulation of the B7x pathway has been shown to prevent or inhibit the development of autoimmune diseases in mouse models and hence potentiation of this pathway could be a therapeutic target for human autoimmune diseases. B7x protein is widely expressed in various cancers and its expression is associated with poor prognostic features in human cancers. B7x has multiple mechanisms of tumorigenesis—decreasing the effector anti-tumor T cells in the tumor, increasing angiogenic factors like VEGF and TGF-B, and activating MDSCs. Blocking B7x in cancer through anti B7x antibodies, CAR T cell or anti-B7x scFV fragments have yielded good results in in vivo cancer models. Hence, B7x represents an attractive therapeutic target in the treatment of cancers and autoimmune diseases.

4 | HHLA2

We and others recently identified HHLA2 as a new member of the B7 family.[2,100,101] It is a type I transmembrane molecule with three extracellular Ig domains.[2] This family usually has one IgV and one IgC extracellular domain (B7-1, B7-2, ICOS-L, PD-L1, PD-L2, and B7x) with an intracytoplasmic tail. B7-H3 is the only member which has a tandem copy of the IgV-IgC extracellular domain resulting in four extracellular domains. The difference in structure with respect to function is currently unknown. The differences could be related to bidirectional signaling of this family or the binding conformations could generate a binding site for another molecule, which could be related to generating a new receptor at the site of their binding.[78]

4.1 | Evolution

HHLA2 is an acronym for Human Endogenous retro virus-H Long terminal repeat-associating 2. This gene was discovered when a systematic search for human retroviral long terminal repeats (LTR) was performed as these LTR's can possess enhancer, promoter, or polyadenylation functions and one such gene identified was HHLA2.[102] The LTR provides the primary polyadenylation signal and hence this integration is important for this gene to be read and translated to mRNA. The LTR of the HHLA2 locus has been integrated into the primate lineage since it is found only in gorilla, chimpanzee, and humans of the New World. HHLA2 is not expressed in mice but it is expressed in higher primates. The integration of the LTR sequence in higher primates could account for the significant differences in gene expression between the different species.

4.2 | Expression

Human Endogenous retro virus-H Long terminal repeat-associating 2 is constitutively expressed on human monocytes and induced on B cells after stimulation with IFN-γ[2] (Table 1). Most normal tissues do not express HHLA2 except the placenta, intestines, kidney, gallbladder, and breast.[103] The placenta is a site of immune privilege and other co-inhibitory molecules namely PD-L1, B7-H3, and B7-x are also highly expressed by this organ.

4.3 | Function

Since HHLA2 belongs to the B7 family, it is hypothesized that HHLA2 regulates T-cell function. HHLA2 immunoglobulin fusion protein (HHLA2-Ig) binds to T cells (resting and activated) and other immune cells demonstrating that there are constitutive receptors on the cell surface. HHLA2-Ig is able to decrease both CD4 and CD8 T-cell proliferation when incubated with anti-CD3. Functionally incubating T cells with HHLA2-Ig decreases the production of several cytokines including IFN-γ, TNF-α, IL-5, IL-10, IL-13, IL-17A, and IL-22.[2] HHLA2 also inhibits IL-2 secretion by T cells in a dose-dependent manner.[104] These experiments demonstrate that HHLA2 inhibits T-cell proliferation and function. HHLA2 also functions as a co-stimulatory molecule and increases cytokine production.[100] It is not uncommon for members of the B7 family to have dual functions depending on the immune milieu, receptor engagement or blockade, or interaction with different receptors[105] (Figure 2). Overall these studies demonstrate that HHLA2 predominantly functions as a T-cell co-inhibitory molecule.

4.4 | HHLA2 in human cancers

Human Endogenous retro virus-H Long terminal repeat-associating 2 is expressed in a majority of tumor specimens of the breast, lung, thyroid, melanoma, ovary, and pancreas.[103] The localization of the protein is both membranous and cytoplasmic in tumor cells. Even though HHLA2 is a transmembrane protein, this type of distribution is not uncommon as this could be due to structural differences in staining vs shuttling of the protein between the cytoplasm and the membrane. HHLA2 protein is also expressed in a lower percentage of other cancers such as liver, bladder, colon, prostate, kidney, and esophagus.[103] The prognostic significance of such an expression was determined in three cancers—breast, osteosarcoma, and lung.

4.4.1 | Breast cancer

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer when compared to estrogen receptor-positive and Her2-positive breast cancer. In a retrospective study, 50 patients with local or locally advanced breast cancer were identified and their clinical characteristics collected. All these patients underwent surgery as the primary treatment followed by chemotherapy or radiotherapy or both. TNBC with a high expression of HHLA2 was associated with lymph node positivity and higher stage at the time of diagnosis, indicating more aggressive disease when compared with tumors with low expression of HHLA2. By analyzing the cBio portal to assess for HHLA2 gene alterations, we found that HHLA2 was altered in 32% of the basal subtype and the vast majority of HHLA2 copy number variations in basal breast cancers were amplifications or gains (>95%). This suggests that one mechanism of upregulation of HHLA2 protein in human cancers could be copy number gain.[103]

4.4.2 | Lung cancer

Tissue microarrays (TMAs) from stage I to III NSCLC specimens from patients undergoing lung cancer resection was constructed from 392 NSCLCs in the discovery cohort tumor tissues and the TMAs in the separate validation cohort consisted of 287 NSCLC cases. Scoring of tumor infiltrating lymphocytes (TILs) as absent, low (1%-30%), or high (>30%) was performed in the same slides which were used for HHLA2 staining.[106] HHLA2 was expressed in 66% of NSCLC while it was not expressed in normal lung tissue including alveolar type 1 and type II cells, endothelial cells and smooth muscle cells in blood vessels. In both the discovery and validation cohort, HHLA2 expression was significantly overexpressed in the EGFR mutant cohort than the WT (89% vs 69% P=.01). In the validation but not the discovery

cohort, HHLA2 positivity was associated with a higher TIL score (86% vs 67% *P*=.03). In the multivariate analysis, a higher TIL score and presence of EGFR mutation were independently associated with HHLA2 expression in lung cancer.[106] Lung cancer is an immunogenic tumor and has variable expression of tumor immune checkpoints. Immunotherapy against PD-1/PD-L1 has been shown to improve survival in patients with disseminated lung cancer. The caveat to this is that only 20% of patients and those who express PD-L1 respond to PD-1/PD-L1 checkpoint blockade. This suggests that other checkpoints like HHLA2 may be more important in the cancers that do not express PD-L1 or in tumors that escape PD-1/PD-L1 blockade.[107]

4.4.3 | Osteosarcoma

Osteosarcoma is another primary malignancy of the bone with a less than 20% 5-year survival rate and the prognostic role of HHLA2 was recently evaluated.[108] In this study, HHLA2 was expressed in metastatic disease more than in primary disease and the differences was statistically significant (93% vs 53%, *P*=.02). Higher levels of HHLA2 expression in the tumor (25% vs 50%) portended a worse OS. Together these studies suggest that HHLA2, by functioning as a T cell co-inhibitory molecule in the tumor microenvironment, could provide a survival advantage for the tumor and hence its expression is associated with a worse prognosis.

4.5 | Summary of HHLA2

Human endogenous retro virus-H Long terminal repeat-associating 2 is a newly discovered T cell immune checkpoint molecule that belongs to the B7 family of ligands. It predominantly functions to inhibit T-cell proliferation and T-cell cytokine responses. HHLA2 is expressed on few normal tissues but it is expressed in various human cancers. High expression of HHLA2 in human cancer of lung, breast, and osteosarcoma is associated with worse prognostic features.

5 | TMIGD2

We and others recently discovered TMIGD2 (transmembrane and immunoglobulin domain containing 2) as one of the receptors for HHLA2 which is a 31-kDa protein and is present on chromosome 19q13.3.[100,103] Sequence analysis showed that TMIGD2, the immunoglobulin-containing and proline-rich receptor-1 (IGPR-1), and CD28 homolog (CD28H) are the same molecule. TMIGD2 (IGPR) was originally identified as an adhesion molecule involved in angiogenesis.[109]

Transmembrane and immunoglobulin domain containing 2 is an Ig superfamily member with an extracellular IgV-like domain, a transmembrane region, and a cytoplasmic tail. The extracellular region has two possible glycosylation sites while the cytoplasmic tail contains tyrosine residues and a proline-rich domain. It shares greater than 10% amino acid identity with other B7 family receptors namely -CD28, CTLA, ICOS, and PD-1.[100] Similar to HHLA2, TMIGD2 is absent in mouse and rat while it is found in higher primates.[109]

TMIGD2 is a membranous glycoprotein which is confirmed by immunofluorescence microscopy. TMIGD2 appears to be in a glycosylated form in the thymus, placenta, heart, small intestine, skin, and kidney with a molecular weight of 55 kDa while in the skeletal muscle, brain, colon, lung, and ovary TMIGD2 seems to be unglycosylated with an apparent molecular weight of 35 kDa.[109]

Transmembrane and Immunoglobulin Domain Containing 2 on its initial discovery as IGPR in cell culture models has been shown to have multiple functions—promote angiogenesis by promoting capillary tube formation of endothelial cells, increases actin filament formation and alters cellular morphology, causes focal adhesion of cells and inhibits cellular migration.[97] TMIGD2 can associate with the SH3 domain containing signaling protein SPIN90 and may play a role in the regulation of angiogenesis.

Transmembrane and Immunoglobulin Domain Containing 2 seems to be preferentially expressed more on lymphoid organs by PCR.[100] TMIGD2 is widely expressed on naive T cells as well as DCs, monocytes, and B cells. Naive CD4 and CD8 αβT cell expressed TMIGD2 while γδT cells and T regulatory cells were negative. However, TMIGD2 is rapidly lost on activation of T cells. TMIGD2 is expressed on naive T cells but is rapidly lost after activation, this is unlikely to be the receptor through which HHLA2 results in a T-cell co-inhibitory function in human cancers. Further research into the expression and role of TMIGD2 protein are needed.

6 | CONCLUSIONS

The B7-CD28 family of ligands and receptors play very important roles in regulating the immune response in normal and pathological states including infection, autoimmunity, and cancer. Blocking the CTLA-4 and the PD-1/PD-L1 axis has directly improved patient survival in metastatic cancer and has underscored the importance of immune checkpoints in cancer. Our understanding of the third group of B7-CD28 members namely B7-H3, B7x, HHLA2, and TMIGD2 has markedly improved, but several questions remain. How do these different immune checkpoints work in tandem? How do we measure the level of T-cell co-stimulation or T-cell co-inhibition rather than viewing it as an 'on-off' state with respect to immune checkpoints? Can the same molecule deliver co-stimulatory and co-inhibitory signals depending on the milieu, interacting receptor, and existence of ligand in more than one state? Some of the challenges with the third group have been: receptor identification, absence of reliable commercial antibodies, and lack of expression of HHLA2 and TMIGD2 in mice. Nevertheless, several in vivo and in vitro studies presented in this review underscore the importance of these new ligands and receptors. Continued research and an improved understanding of these pathways can lead to successful therapeutic applications in human autoimmune diseases and cancer.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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